THERAPEUTIC APPLICATION OF CHIMERIC AND RADIO-LABELLED
ANTIBODIES TO HUMAN B LYMPHOCYTE RESTRICTED DIFFERENTIATION ANTIGEN FOR TREATMENT OF B CELL LYMPHOMA

Inventors: Darrell R. Anderson, Escondido; Nabil Hanna, Ovivenuhain; John E. Leonard, Escondido; Roland A. Newman; Mitchell E. Reff, both of San Diego; William H. Rastetter, Rancho Santa Fe, all of Calif.

Assignee: Idec Pharmaceuticals Corporation, San Diego, Calif.

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Field of Search \[424/133.1, 143.1, 424/144.1, 156.1, 174.1, 800, 801, 153.1, 435/69.6, 172.3, 252.3, 320.1, 240.2, 328, 334, 343.1, 536/23.4, 23.5, 23.53, 530/387.3, 388.22, 388.73, 388.85, 867; 935/70; 72, 15, 104

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Lipton, N.M. "60/1 Blood 170a (Abs. 609) (1982).

Primary Examiner—Ronald B. Schwadron
Attorney, Agent, or Firm—Burns, Doane, Swecker & Mathis, LLP

ABSTRACT

Disclosed herein are therapeutic treatment protocols designed for the treatment of B cell lymphomas. These protocols are based upon therapeutic strategies which include the use of administration of immunologically active mouse/human chimeric anti-CD20 antibodies, radiolabeled anti-CD20 antibodies, and cooperative strategies comprising the use of chimeric anti-CD20 antibodies and radiolabeled anti-CD20 antibodies.

6 Claims, 21 Drawing Sheets
OTHER PUBLICATIONS


TGTTCCCCCT CCCCCGTTGCT TCTCTTGACC GTGGAAAGTG CCACTCCCACT TGTCCCTTCC
BGH poly A=231bp

TAAATAAAATGC AGGAAATTGAC AAGCAGATTGT CTGAGTAAAG GTCATCTCTAT TCTGGGGGGG
GGGTGGGGGTC AGGAGACGAA CCGGGGAGAT TGGGAAAGAC ATAGAGAGCA TCGTGGGGA
GGGTTGGGCT CTATGGAAACC AAGCTTGGGGCT CGAACACATAT GCGAAGTAGCC CCCCCATTTG

ACCTGAATAG CGCTAATAGG CCGGCTCTGTC ATTATGCCCA GTACATGACC TTATGGGACT
TTCTACTTTG GCAGTACATC TACTATTAG TCATTGCGAT TACCATGAGT ATGGGCTTTT
CMV PROMOTER-ENHANCER=334bp
GCGAGTCATG CAATGGCGCT GGTAGACGGT TTAGAATCAG GGGATTLELA AGTCTCCACC
CCATTGACGT CAATGGGAGT TTTGTTTGGC ACCAAAATAC ACGGGACTTTT CCAAAAATGT
GATAACACTG CCCCCATTGG AGCAGAAATTT GCGGTAGGGGCT GTGACGGGTC AGGTCTTATA

LINKER #6=7bp
TAAGCAGGAG CTGGTACCTTC CTCACATCCA GTGATCAAGCA GTGAACACAG ACCGGTGCA

Sal I
LEADER=51bp

START HEAVY CHAIN

AAGGGCCCAG CGGTCTTCCG CCGGGAGCGCT TCTCGTCAACA GACCTCTGG GGGCACAGCG
GGCTGGGGGT CCGCTTCGAC CAACATCTTC CCGGAACGGG AGGCTGTTGC GTGAAACTCA
GGGGCCCTGA CCAAGGGGGT CCAACACCTG CGGGCTGTCG AACAGCTCAG AGGACTCTAC

HUMAN GAMMA 1 CONSTANT

TGCTCAGCA CGGTGGGTAG CGCTGCCCTCC AGGCTGGTTCTG GCACCCAGAC ATACATCTGC

993bp=230 AMINO ACID & STOP CODON
AACGTGAATC ACAAGCCCAA CAACAAGAAAG GTGAGCAAGAA AAGCAGAGCC CAATCTTGT
GCAAAAACTC ACAACATGCGG ACCTGCGACA GCACTCTGAC TCCCTGGGGG ACCGTGACT
TTCTCTTCC CCCCCAACCA AAGGAGCCAC CTTATGACAC CCGGAACGTC TGGAGTCAAA
TGCGTGGTGG TGAGCAATCA CGCGGAGAGG CGCTGGGATG ACTGATAAGC ATGGTCGAC
GGCGTGGAGG TGCTTAAAG CGAGAAGAGG CCGGCGGAGG AGGAGTACCA CAGCAGATAC
CGTGTGGTCA GGCCTTCTAC CGTGTGTGAC CAGGAGCTGG TCAGATGGCA GGACTACAG
TGCAAGGTTT CCCAGAAGCG CTCTCCAGCG CCAATGGAGA AAACGATCTC CAACAGCCAA
GGCGACGGC GAGAACCACA GGCTGATCC CTGGGGCGCT CGCGGATGA GTGACCAGG
AAGCAGGTCA GCCTGACCTG CTGGTCTAAA GGGTTCTATC CCAGGCGAC AGGCTGGGAC
TGCGAGAGCA ATGGGCGACGC GGAGAAGACG TACAAAGGCA CGGCTCCGCT GTGGGACTCC

FIG. 2B
FIG. 2C
<table>
<thead>
<tr>
<th>Linker #10 = 10bp</th>
<th>Stop DHFR</th>
<th>3' Untranslated DHFR = 82bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linker #11 = 10bp</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Linker #12 = 21bp</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Start Neo</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Mouse Beta Globin Major Promoter = 369bp**

**Neo**

**Phosphotransferase**

**Amino Acids & Stop Codon**

**FIG. 2D**
STOP NEO
6043.1

GAAATGACCG ACCAAGCCGAC GCCCAACCTG CCCATACCGG ATTTGAGATTCCAGGACCGG

STOP NEO=173bp
2

TTCTATGAAA GGTGGACTTT CCAATCTGGCC TTCCGCGAGT CCGCTGGAAT GATCCCTCCAS

6180

CGCCGGGATC TCATGCTGGA GTCTTCGCCC AACCCTAALT TTGTTATTGCG AGCTTATAAT

6240

SG7-TACAATA AAGCAATAG CATCACAAT TTTCAATATAA AAGCATTTTT TTTAATTCGAC

6300

SV40 POLY A EARLY=133bp

6360

TCTAGTTTGT GTTTGTCGCA AATCATCAGT CTATTTTTAT CGGCTGCCCGG

6420

ATCCGGGCTA GAGCTGGGCG TAAATCATCT TATCATGCTT TCTCTGTTGA AATTGTATTAT

6480

CGCTCACAAT TCCACACAA ATACAAGCCG GAAAGCTAAA GTGTAAGGCC TGGGTGGCCT

6540

AATCTAGTAA TAACTCACA TTAATTTGCT TGCCGTTACCT CCGCCGCTTC CAGCAGCGAA

6600

ACCTGTCTGT CGAGCTGCTA TAATCTCTGCT CCGCCCCCGC GGGAGAGCGG GGTTCCTGGTA

6660

TTGCCGGGCTC TTCCGGTTCC TCAGGCTGGC ATCTGCTGGC CTCGGCTGGTT CGGCTGCCGGC

6720

GAGCGGTATC AGCTCACGCA AAGCGGGGAA TACGGTTTATC CACAGATACA GGGGATAAGC

6780

CAGCGAAAGA CATATGAGCA AAGGCCAGCC AAGAGCCAGG GAAGCTGAAA AAGGGCCGCT

6840

6792=BACTERIAL ORIGIN OF REPLICATION

TGGCGGGCTT TCCCATAGG CTCGCCCCC CTTGACGAGCA TTAGGAAATA GTACGCCCTAG

5840

GTCGGAGGTC GGCAAGCCCG AGAGGACTAT AACATACACG GGGGTTTCCC CTTGGAGCCT

5900

CCTCGGTGCG GCTCGCCTGG CCGACCCTCGC CCGTTACCAG CACACGGTCC GCTTTCTCC

5960

CTTCGGCAAG CCGGCGGCTT TCTCAATGCT CAGCCTGCTAG CTAATCGTAG TGGGATGAGG

6020

TCTGCTGCCA CAAGCTGGGC TGTGCTGGAC AAGCGGGGCG TCAAGGGCGC GCGGCTGCGC

6080

TATCGGGTAA CTATCGTCTT AGATCCAAAC CGATAAGACA CGACTATATCC AACCTGGCAG

6140

CAGCCACTTG TAAAGGAGTA GAGCAGGAGA GCTATGGAAC CGGCTGCTAC GAGTTCTTTA

6200

AGCTGGTGCC TAACTACGCC TACACTAGGA GGACTAGTATG TGGTAAGCTTC GCTCGCTGAG

6260

AGGCAGTTAC CTCGCGGAAA AGAGTTGTAA GCTCGTGTAC CCGCAACGAA ACCACCGGCT

6320

GTACGGGCTG GTTTTTTTGTT TGCAAGCGGCA ATAGTACGCC CAGAAAAAAA GATCTCAAGG

6380

AAGATCTTTT GATCTTTTTT ACCGGGCTCTG AACCCTGACT GACGCCGAGT GACATTTAA

6440

GGAT'TTTGGT CATGAGGT AGCAGAAGGA TCTCTTGTTT GCATGTTTTAT AATTTAAAAAT

6500

FIG. 2E
STOP BETA LACTAMASE

GAAGTTTTAA ATCAATCTAA AGTATATATG AGTAAACTTG GTCGTGAGCT
TAATCATGA GGGACTATTC TCGCCGATCT GTCTATTCCG TTCAATTCGAA GTCGCTGAC
TCCCCGTCTG TAGATAACT ACGATAACGG AGGCTTTACG ATCTGGCCCG AGTCTGCAA
TGATACCGCT AGACCCACCTTC TACCCGCTTC CAGATTATGC AGCAATAAAC CATCCACCGCG
GAAGGGCCGA CGCCAGAAGT GTCCCGCGAA GCTATCTCCGAC TCTATCCAG TCTATATT

BETA LACTAMASE=861 bp

286 AMINO ACID & STOP CODON

GTTGCGGGA AGCTAGAGTA AGTAGTTTGC CAGTAAATAG TGGCGCAAC GTTGTGCGCA
TTGCTACAGG CATCGTGCGT TCGCGCTCTG CTTTCGATTG GCGTTGATTG AGCCTCGGTT
CCCAACGATC AAGGGCGAGTT ACATGATCCCC CCAAGGTTGG CAAAAAAGCG GTTACCCTCC
TGGTCTCCCG GATCGTGTGC AAGAAGTAACT TGGGCGGGAT GTTATCTGATG ATGTTATTGG

CAGCACTGCA TAAATCTCTTT ACTGTCAATGC CACTCGTAAG ATGCTTTCCT GTGACTGGTG
AGTACTCAAC CAAGCTATTC TGAGAAATAG GTATCCGGGG ACCGGTGTGC TCTTGCCCGG
CGCAGATACG GGAATATCC GCAGCACATA GCAACACCTT AAAAGTGCTC ATCATGGGAA
AACGTTCTTC GGGCGGAAA CCCCTCAAGGA CTCACCGGCT GTGAGACGT AGTCTAGGG
AACCCACTGG TCGCCACCAAC TGATACTGAG CATCTTTAAC TTTCAACCGGC GTTCTGGGT
CGGAAAAAC AGGAAAGCAGAA TATGCCGCAA AAAAAAGGAA AAGGGCCAGGA CGGAAATTT

START BETA LACTAMASE

GAATACATGCT GTCTTTCTTT TTCTCAGATTT ATGAAGGAAT TTACGAGGT TATCTCCTCA

TTACCGGATA CATATTTGAA GTATTATGAA AAAAAATAAACA AATAGGGGTTT CGGCCCCAAC

FIG. 2F
HUMAN KAPPA CONSTANT=324bp=107 AMINO ACID & STOP CODON
CTCCAAATCGG GTAACCCTCA GGAAGGCTC AGCAGGCGAG ACAGCAAGGA CAGGACCTAC 1560
ACGCCATCAG AGCACCCTGAC CCTGACCAAA GGCAAGTACG AGGAAACAAAC AGAITCTACGC 1620
TGGCGAAGTCA CCCATCAGGG CCTGAGCTCG CCGGTACCAAA AGAGCTCAAAG CAGGGGAGAG 1680
STOP
LIGHT
CHAIN Eco RI

LINKER #4=81bp
TATGGATCTTAGATCCGTAAAGCTGCGTTACTACAG

BOVINE GROWTH HORMONE POLYADENYLATION REGION=231bp

ECO RI LINKER #5=15bp

CMV PROMOTER-ENHANCER=334bp

HEAVY CHAIN SYNTHETIC & NATURAL LEADER
Sal I

FIG. 3B
STOP HEAVY CHAIN Frame HI: LINKER #7=81bp
CAGGAAGGCC TCTCCCTGTC TCGGGCTAAA TCGACGATTG TTAACGCTTA CGAAGACTCT 3840
AGACGGAT CATGACCAACT GGGCGCCCTG ATATCTAGCT ATATCTAGCT TCGACGATTG 3900
CTCTAGATG CCAAGATCAG GTTGTTCCT CCTCCGCGCT CCTCTCTTTC ACCCTGGAGA 3960
GTCGACCT CGACTGCTTT TCTGATTAT AATGAGAAT TGGTGGAGAT TGCTGAGTA 4020
BOVINE GROWTH HORMONE POLYADENYLATION (REGION=291bp)
GCTGCGCATT ATATCTGGGG GGTGGGGGGG GCAGGGACAC CAAAGGGGGG GAGGGGGGAG 4080
[LINKER #6=34bp]
ACAATGAGAC GGATGGCTGG GATGCGGTGG GCTCTATAGA ACCAGCTGGA TGGCGACAGC 4140
GTCGACCT CGACTGCTTT TCTGATTAT AATGAGAAT TGGTGGAGAT TGCTGAGTA 4125
MOUSE BETAGLOBIN MAJOR PROMOTER=366bp
ATGCATTTAGA GACAGTGGTC TCTGCGCAAG TAAGGACAAA CATTATCGA ATGGAGACTG 4320
CAGACCGTAG ACTCTAAGAC CAGTGAGTGG CACAGCATTTC AAGGGAGAAA TAGCGCTGTC 4380
ATCAACCGAG CCGATGGCG TAAAGCCACA CTTGGGTAAG GGGCAATCTG TCTACACAGG 4440
ATAGCAAGGG CAGGACGGCC AGGCAACGAT ATAGGTCGAG TGAGGATCAG TGCTGCTCTCA 4500

FIG. 3C
FIG. 3E
AGTCATGAG TCGAGCTGGG CAGAACCCAG TACGGGAAA TACATCAGT AAGATACAG GCGGTTTCCC W560 TAC CTCTCC 7 S2O TATCTCA (T 680 CAGCCC (, , , 740 (ACTATCC 780O (CTOCTACAC 860 (TACT (CC 7920 (CCAAACAAA 798O AAAAAAAA ( 8 C-40 AACEAAA CT 8 O ATCCTT AA STOP TCTGACAGIT 2.20 R 16) T CATCC AT AG, 828O TCT (CCCC (A 85.40 84 (OO) AMINO AC) & STOP CODON CTA AC GA Ti (CC ATTAAT ACT CAii CT (TTTCC. A A ... CT CAA CAT CTTACCGCT. A TC T CA; C (, TCTTTT ACT ACCCGC AAA AAA ART BETA LACTAMASE - - - - - AATAA TGAAC CAT CAT ACAA AAA

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Apr. 7, 1998
Sheet 13 of 21
5,736,137

GACGCTCAAG TCAAGGGTGC CGAACCACCGA CAGGACTATA AAGATACAG GCGGTTTCCC W560 TAC CTCTCC 7 S2O TATCTCA (T 680 CAGCCC (, , , 740 (ACTATCC 780O (CTOCTACAC 860 (TACT (CC 7920 (CCAAACAAA 798O AAAAAAAA ( 8 C-40 AACEAAA CT 8 O ATCCTT AA STOP TCTGACAGIT 2.20 R 16) T CATCC AT AG, 828O TCT (CCCC (A 85.40 84 (OO) AMINO AC) & STOP CODON CTA AC GA Ti (CC ATTAAT ACT CAii CT (TTTCC. A A ... CT CAA CAT CTTACCGCT. A TC T CA; C (, TCTTTT ACT ACCCGC AAA AAA ART BETA LACTAMASE - - - - - AATAA TGAAC CAT CAT ACAA AAA

FIG. 3F
FIG. 4
FIG. 5
FIG. 6

FIG. 7
FIG. 8

FIG. 9A
% OF PRE-EXISTING B CELLS

1.6mg/kg DOSE
- SP2/0
- CHO

DAYS POST INFUSION

120
100
80
60
40
20
0

0 1 3 7 14 21

FIG. 9B

% OF PRE-EXISTING B CELLS

6.4mg/kg DOSE
- SP2/0
- CHO

DAYS POST INFUSION

120
100
80
60
40
20
0

0 1 3 7 14 21

FIG. 9C
**FIG. 10**

Graph showing the percent remaining B cells over time (days).

**FIG. 11**

Graph showing the tumor size (square mm) over time (days) for saline and Y2B8 treatments.
**FIG. 12**

- **SALINE**
- **C2B8**

**FIG. 13**

- **SALINE**
- **Y2B8+C2B8**
**FIG. 14A**

100 mg/m²: PR

**FIG. 14B**

500 mg/m²: MR
THERAPEUTIC APPLICATION OF CHIMERIC AND RADIO-LABELED ANTIBODIES TO HUMAN B LYMPHOCYTE RESTRICTED DIFFERENTIATION ANTIGEN FOR TREATMENT OF B CELL LYMPHOMA

RELATED APPLICATIONS

This is a Continuation-in-Part of U.S. Ser. No. 07/977,891, filed Nov. 13, 1992, now abandoned. This patent document is related to U.S. Ser. No. 07/977,691 (now abandoned), entitled "IMPAIRED DOMINANT SELECTABLE MARKER SEQUENCE FOR ENHANCEMENT OF EXPRESSION OF CO-LINKED GENE PRODUCT AND EXPRESSION VECTOR SYSTEMS COMPRISING SAME" having U.S. Ser. No. 07/977,691 (now abandoned; filed Nov. 13, 1992) and "IMPAIRED DOMINANT SELECTABLE MARKER SEQUENCE AND INTRONIC INSERTION STRATEGIES FOR ENHANCEMENT OF EXPRESSION OF GENE PRODUCT AND EXPRESSION VECTOR SYSTEMS COMPRISING SAME." U.S. Ser. No. 08/147,696 (now U.S. Pat. No. 5,648,267) (filed simultaneously herewith). The related patent documents are incorporated herein by reference.

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   ii. Phase I/II Clinical Trial of 2B8: Multiple Dose Therapy Study
IV. COMBINATION THERAPY: 2B8 AND Y2B8

A. Field of the Invention

The references to be discussed throughout this document are set forth merely for the information described therein prior to the filing dates of this document, and nothing herein is to be construed as an admission, either express or implied, that the references are "prior art" or that the inventors are not entitled to annul such descriptions by virtue of prior inventions or priority based on earlier filed applications.

The present invention is directed to the treatment of B cell lymphoma using chimeric and radiolabeled antibodies to the B cell surface antigen Bp35 ("CD20").

B. Background of the Invention

The immune system of vertebrates (for example, primates, which include humans, apes, monkeys, etc.) consists of a number of organs and cell types which have evolved to: accurately and specifically recognize foreign microorganisms ("antigen") which invade the vertebrate-host; specifically bind to such foreign microorganisms; and, eliminate/destroy such foreign microorganisms. Lymphocytes, amongst others, are critical to the immune system. Lymphocytes are produced in the thymus, spleen and bone marrow (adult) and represent about 30% of the total white blood cells present in the circulatory system of humans (adult). There are two major sub-populations of lymphocytes: T cells and B cells. T cells are responsible for cell mediated immunity, while B cells are responsible for antibody production (humoral immunity). However, T cells and B cells can be considered as interdependent—in a typical immune response, T cells are activated when the T cell receptor binds to fragments of an antigen that are bound to major histocompatibility complex ("MHC") glycoproteins on the surface of an antigen presenting cell; such activation causes release of biological mediators ("interleukins") which, in essence, stimulate B cells to...
5,7-3 differentiate and produce antibody ("immunoglobulins") against the antigen.

Each bone marrow in the host expresses a different antibody on its surface—thus, one B cell will express antibody specific for one antigen, while another B cell will express antibody specific for a different antigen. Accordingly, B cells are quite diverse, and this diversity is critical to the immune system. In humans, each B cell can produce an enormous number of antibody molecules (i.e., about $10^7$ to $10^8$). Such antibody production most typically ceases (or substantially decreases) when the foreign antigen has been neutralized. Occasionally, however, proliferation of a particular B cell will continue unabated; such proliferation can result in a cancer referred to as "B cell lymphoma."

T cells and B cells both comprise cell surface proteins which can be utilized as "markers" for differentiation and identification. One such human B cell marker is the human B lymphocyte-restricted differentiation antigen Bp35, referred to as "CD20." CD20 is expressed during early pro-B cell development and remains until plasma cell differentiation. Specifically, the CD20 molecule may regulate a step in the activation process which is required for cell cycle initiation and differentiation and is usually expressed at very high levels on neoplastic ("tumor") B cells. CD20, by definition, is present on both "normal" B cells as well as "malignant" B cells, i.e., those B cells whose unabated proliferation can lead to B cell lymphoma. Thus, the CD20 surface antigen has the potential of serving as a candidate for "targeting" of B cell lymphomas.

In essence, such targeting can be generalized as follows: antibodies specific to the CD20 surface antigen of B cells are, e.g., injected into a patient. These anti-CD20 antibodies specifically bind to the CD20 cell surface antigen of (ostensibly) both normal and malignant B cells; the anti-CD20 antibody bound to the CD20 surface antigen may lead to the destruction and depilation of neoplastic B cells. Additionally, chemical agents or radioactive labels having the potential to destroy the tumor can be conjugated to the anti-CD20 antibody such that the agent is specifically "delivered" to, e.g., the neoplastic B cells. Irrespective of the approach, a primary goal is to destroy the tumor: the specific approach can be determined by the particular anti-CD20 antibody which is utilized and, thus, the available approaches to targeting the CD20 antigen can vary considerably.

For example, attempts at such targeting of CD20 surface antigen have been reported. Murine (mouse) monoclonal antibody IF5 (an anti-CD20 antibody) was reportedly administered by continuous intravenous infusion to B cell lymphoma patients. Extremely high levels (>2 grams) of IF5 were reportedly required to deplete circulating tumor cells, and the results were described as being "transient." Press et al. "Monoclonal Antibody IF5 (Anti-CD20) Scero-therapy of Human B-Cell Lymphomas." Blood 69(2):584–591 (1987). A potential problem with this approach is that non-human monoclonal antibodies (e.g., murine monoclonal antibodies) typically lack human effector functionality, i.e., they are unable to, inter alia, mediate complement dependent lysis or lyse human target cells through antibody dependent cellular toxicity or Fc-receptor mediated phagocytosis. Furthermore, non-human monoclonal antibodies can be recognized by the human host as a foreign protein; therefore, repeated injections of such foreign antibodies can lead to the induction of immune responses leading to harmful hypersensitivity reactions. For murine-based monoclonal antibodies, this is often referred to as a Human Anti-Mouse Antibody response, or "HAMA" response. Additionally, these "foreign" antibodies can be attacked by the immune system of the host such that they are, in effect, neutralized before they reach their target site.

Lymphocytes and lymphoma cells are inherently sensitive to radiotherapy for several reasons: the local emission of ionizing radiation of radiolabeled antibodies may kill cells with or without the target antigen (e.g., CD20) in close proximity to antibody bound to the antigen; penetrating radiation may obviate the problem of limited access to the antibody in vivo. The authors have calculated that the total amount of antibody required may be reduced. The radionuclide emits radioactive particles which can damage cellular DNA to the point where the cellular repair mechanisms are unable to allow the cell to continue living; therefore, if the target cells are tumors, the radioactive label beneficially kills the tumor cells. Radiolabeled antibodies, by definition, include the use of a radioactive substance which may require the need for precautions for both the patient (e.g., possible bone marrow transplantation) as well as the health care provider (i.e., the need to exercise a high degree of caution when working with the radioactivity).


Toxins (i.e., chemotherapeutic agents such as doxorubicin or mitomycin C) have also been conjugated to antibodies. See, for example, PCT published application WO 92/07466 (published May 14, 1992).

"Chimeric" antibodies, i.e., antibodies which comprise portions from two or more different species (e.g., mouse and human) have been developed as an alternative to "conjugated" antibodies. For example, Liu, A. Y., et al. "Production of a Mouse-Human Chimeric Monoclonal Antibody to CD20 with Potent Fe-Dependent Biologic Activity" J. Immun. 139(10):3521–3526 (1987), describes a mouse human chimeric antibody directed against the CD20 antigen. See also, PCT Publication No. WO 89/04936. However, no information is provided as to the ability, efficacy or practicability of using such chimeric antibodies for the treatment of B cell disorders in the reference. It is noted that in vitro
functional assays (e.g., complement dependent lysis ("CDC"); antibody dependent cellular cytotoxicity ("ADCC"), etc.) cannot inherently predict the in vivo capability of a chimeric antibody to destroy or deplete target cells expressing the specific antigen. See, for example, Robinson, R. D. et al., "Chimeric mouse-human anti-carcinoma antibodies that mediate different anti-tumor cell biological activities." *Hum. Antibod. Hybridomas* 2:84–93 (1991) (chimeric mouse-human antibodies having undetectable ADCC activity). Therefore, the potential therapeutic efficacy of chimeric antibody can only truly be assessed by in vivo experimentation.

What is needed, and what would be a great advance in the art, are therapeutic approaches targeting the CD20 antigen for the treatment of B cell lymphomas in primates, including, but not limited to, humans.

C. SUMMARY OF THE INVENTION

Disclosed herein are therapeutic methods designed for the treatment of B cell disorders, and in particular, B cell lymphomas. These protocols are based upon the administration of immunologically active chimeric anti-CD20 antibodies for the depletion of peripheral blood B cells, including B cells associated with lymphomas; administration of radiolabeled anti-CD20 antibodies for targeting localized and peripheral B cell associated tumors; and administration of chimeric anti-CD20 antibodies and radiolabeled anti-CD20 antibodies in a cooperative therapeutic strategy.

D. BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a diagrammatic representation of a tandem chimeric antibody expression vector useful in the production of immunologically active chimeric anti-CD20 antibodies ("TCAE 8");

FIGS. 2A through 2F are the nucleic acid sequence (SEQ. ID. NO. 2) of the vector of FIG. 1;

FIGS. 3A through 3F are the nucleic acid sequence (SEQ. ID. NO. 3) of the vector of FIG. 1 further comprising murine light and heavy chain variable regions ("anti-CD20 in TCAE 8");

FIG. 4 is the nucleic acid and amino acid sequences (SEQ. ID. NO. 6) (including CDRI and framework regions) of murine variable region light chain derived from murine anti-CD20 monoclonal antibody 2B8;

FIG. 5 is the nucleic acid and amino acid sequences (SEQ. ID. NO. 9) (including CDRI and framework regions) of murine variable region heavy chain derived from murine anti-CD20 monoclonal antibody 2B8;

FIG. 6 are flow cytometry results evidencing binding of fluorescent-labeled human Clq to chimeric anti-CD20 antibody, including, as controls labeled Clq; labeled Clq and murine anti-CD20 monoclonal antibody 2B8; and labeled Clq and human IgG1k;

FIG. 7 represents the results of complement related assays comparing chimeric anti-CD20 antibody and murine anti-CD20 monoclonal antibody 2B8;

FIG. 8 represents the results of antibody mediated cellular cytotoxicity assay in vivo human effector cells comparing chimeric anti-CD20 antibody and 2B8;

FIGS. 9A. 9B and 9C provide the results of non-human primate peripheral blood lymphocyte depletion after infusion of 0.4 mg/kg (A); 1.6 mg/kg (B); and 6.4 mg/kg (C) of immunologically active chimeric anti-CD20 antibody;

FIG. 10 provides the results of, inter alia, non-human primate peripheral blood lymphocyte depletion after infusion of 0.01 mg/kg of immunologically active chimeric anti-CD20 antibody;

FIG. 11 provides results of the tumoricidal impact of Y2B8 in a mouse xenographic model utilizing a B cell lymphoblastic tumor;

FIG. 12 provides results of the tumoricidal impact of C2B8 in a mouse xenographic model utilizing a B cell lymphoblastic tumor;

FIG. 13 provides results of the tumoricidal impact of a combination of Y2B8 and C2B8 in a mouse xenographic model utilizing a B cell lymphoblastic tumor; and

FIGS. 14A and 14B provide results from a Phase I/II clinical analysis of C2B8 evidencing B-cell population depletion over time for patients evidencing a partial remission of the disease (14A) and a minor remission of the disease (14B).

E. DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

Generally, antibodies are composed of two light chains and two heavy chain molecules; these chains form a general "Y" shape, with both light and heavy chains forming the arms of the Y and the heavy chains forming the base of the Y. Light and heavy chains are divided into domains of structural and functional homology. The variable domains of both the light ("V_L") and the heavy ("V_H") chains determine recognition and specificity. The constant region domains of light ("C_L") and heavy ("C_H") chains confer important biological properties, e.g. antibody chain association, secretion, transplacental mobility, Fc receptor binding complement binding, etc. The series of events leading to immunoglobulin gene expression in the antibody producing cells are complex. The variable domain region gene sequences are located in separate germ line gene segments referred to as "V_Y", "D_Y", and "J_Y", or "V_L" and "J_L." These gene segments are joined by DNA rearrangements to form the complete V regions expressed in heavy and light chains, respectively. The rearranged, joined V segments (V_L-J_L and V_H-D-J_H) then encode the complete variable regions or antigen binding domains of light and heavy chains, respectively.

Serotherapy of human B cell lymphomas using an anti-CD20 murine monoclonal antibody (1F5) has been described by Press et al., (69 Blood 584, 1987, supra); the reported therapeutic responses, unfortunately, were transient. Additionally, 25% of the tested patients reportedly developed a human anti-mouse antibody (HAMA) response to the serotherapy. Press et al., suggest that these antibodies, conjugated to toxins or radioisotopes, might afford a more lasting clinical benefit than the unconjugated antibody.

Owing to the debilitating effects of B cell lymphoma and the very real need to provide viable treatment approaches to this disease, we have embarked upon different approaches having a particular antibody, 2B8, as the common link between the approaches. One such approach advantageously exploits the ability of mammalian systems to readily and efficiently recover peripheral blood B cells; using this approach, we seek to, in essence, purge or deplete B cells in peripheral blood and lymphatic tissue as a means of also removing B cell lymphomas. We accomplish this by utilization of, inter alia, immunologically active, chimeric anti-CD20 antibodies. In another approach, we seek to target tumor cells for destruction with radioactive labels.

As used herein, the term "anti-CD20 antibody" is an antibody which specifically recognizes a cell surface nonglycosylated phosphoprotein of 35,000 Daltons, typically
designated as the human B lymphocyte restricted differentiation antigen Bp35, commonly referred to as CD20. As used herein, the term "chimeric" when used in reference to anti-CD20 antibodies, encompasses antibodies which are most preferably derived using recombinant deoxyribo-nucleic acid techniques and which comprise both human (including immunologically "related" species, e.g., chimpanzee) and non-human components; the constant region of the chimeric antibody is most preferably substantially identical to the constant region of a natural human antibody; the variable region of the chimeric antibody is most preferably derived from a non-human source and has the desired antigenic and specificity to the CD20 cell surface antigen. The non-human source can be any vertebrate source which can be used to generate antibodies to a human CD20 cell surface antigen or material comprising a human CD20 cell surface antigen. Such non-human source includes, but is not limited to, rodents (e.g., rat, mouse, etc.) and non-human primates (e.g., Old World Monkey, Ape, etc.). Most preferably, the non-human component (variable region) is derived from a murine source. As used herein, the phrase "immunologically active" when used in reference to chimeric anti-CD20 antibodies, means a chimeric antibody which binds human Clq, mediates complement dependent lysis ("CDC") of human B lymphoid cell lines, and lyses human target cells through antibody dependent cellular cytotoxicity ("ADCC"). As used herein, the phrases "indirect labeling" and "indirect labeling approach" both mean that a chelating agent is covalently attached to an antibody and at least one radionuclide is inserted into the chelating agent. Preferred chelating agents and radionuclides are set forth in Srivagava, S. C. and Mease, R. C., "Progress in Research on Ligands, Nuclides and Techniques for Labeling Monoclonal Antibodies." *Nucl. Med. Bio.* 18/6: 589-603 (1991) ("Srivagava") which is incorporated herein by reference. A particularly preferred chelating agent is 1-isothiocyanato-2-methylimidazole tris (semicarbazone) acetate ("MX-DTPA"); particularly preferred radionuclides for indirect labeling include indium [111] and yttrium [90]. As used herein, the phrases "direct labeling" and "direct labeling approach" both mean that a radionuclide is covalently attached directly to an antibody (typically via an amino acid residue). Preferred radionuclides are provided in Srivagava; a particularly preferred radionuclide for direct labeling is iodine [131] covalently attached via tyrosine residues. The indirect labeling approach is particularly preferred.

The therapeutic approaches disclosed herein are based upon the ability of the immune system of primates to rapidly recover, or rejuvenate, peripheral blood B cells. Additionally, because the principal immune response of primates is occasioned by T cells, when the immune system has a peripheral blood B cell deficiency, the need for "extraordinary" precautions (i.e. patient isolation, etc.) is not necessary. As a result of these and other nuances of the immune systems of primates, our therapeutic approach to B cell disorders allows for the purging of peripheral blood B cells using immunologically active chimeric anti-CD20 antibodies.

Because peripheral blood B cell disorders, by definition, can indicate a necessity for access to the blood for treatment, the route of administration of the immunologically active chimeric anti-CD20 antibodies and radioiodinated anti-CD20 antibodies is preferably parenteral; as used herein, the term "parenteral" includes intravenous, intramuscular, subcutaneous, rectal, vaginal or intraperitoneal administration. Of these, intravenous administration is most preferred.

The immunologically active chimeric anti-CD20 antibodies and radioiodinated anti-CD20 antibodies will typically be provided by standard technique within a pharmaceutically acceptable buffer, for example, sterile saline, sterile buffered water, propylene glycol, combinations of the foregoing, etc. Methods for preparing parenterally administrable agents are described in *Pharmaceutical Carriers & Formulations*, Martin, Remington's Pharmaceutical Sciences, 15th Ed. (Mack Pub. Co., Easton, Pa. 1975), which is incorporated herein by reference.

The specific, therapeutically effective amount of immunologically active chimeric anti-CD20 antibodies useful to produce a unique therapeutic effect in any given patient can be determined by standard techniques well known to those of ordinary skill in the art. Effective dosages (i.e., therapeutically effective amounts) of the immunologically active chimeric anti-CD20 antibodies range from about 0.001 to about 30 mg/kg body weight, more preferably from about 0.01 to about 25 mg/kg body weight, and most preferably from about 0.4 to about 20.0 mg/kg body weight. Other dosages are viable; factors influencing dosage include, but are not limited to, the severity of the disease; previous treatment approaches; overall health of the patient; other diseases present, etc. The skilled artisan is readily credited with assessing a particular patient and determining a suitable dosage that falls within the ranges, or if necessary, outside of the ranges.

Introduction of the immunologically active chimeric anti-CD20 antibodies in these dose ranges can be carried out as a single treatment or over a series of treatments. With respect to chimeric antibodies, it is preferred that such introduction be carried out over a series of treatments; this preferred approach is predicated upon the treatment methodology associated with this disease. While not wishing to be bound by any particular theory, because the immunologically active chimeric anti-CD20 antibodies are both immunologically active and bind to CD20, upon initial introduction of the immunologically active chimeric anti-CD20 antibodies to the individual, peripheral blood B cell depletion will begin; we have observed a nearly complete depletion within about 24 hours post treatment infusion. Because of this, subsequent introduction(s) of the immunologically active chimeric anti-CD20 antibodies (or radioiodinated anti-CD20 antibodies) to the patient is preferred to: a) clear remaining peripheral blood B cells; b) begin B cell depletion from lymph nodes; c) begin B cell depletion from other tissue sources, e.g., bone marrow, tumor, etc. Stated again, by using repeated introductions of the immunologically active chimeric anti-CD20 antibodies, a series of events take place, each event being viewed by us as important to effective treatment of the disease. The first "event" then, can be viewed as principally directed to substantially depleting the patient's peripheral blood B cells; the subsequent "events" can be viewed as either principally directed to simultaneously or serially clearing remaining B cells from the system clearing lymph node B cells, or clearing other tissue B cells.

In effect, while a single dosage provides benefits and can be effectively utilized for disease treatment/management, a preferred treatment course can occur over several stages; most preferably, between about 0.4 and about 20 mg/kg body weight of the immunologically active chimeric anti-CD20 antibodies is introduced to the patient once a week for between about 2 to 10 weeks, most preferably for about 4 weeks.

With reference to the use of radioiodinated anti-CD20 antibodies, a preference is that the antibody is non-chimeric;
this preference is predicted upon the significantly longer circulating half-life of chimeric antibodies vis-a-vis murine antibodies (ie, with a longer circulating half-life, the radionuclide is present in the patient for extended periods). However, radiolabeled chimeric antibodies can be beneficially utilized with lower milli-Curies ("mCi") dosages used in conjunction with the chimeric antibody relative to the murine antibody. This scenario allows for a decrease in bone marrow toxicity to an acceptable level, while maintaining therapeutic utility.

A variety of radionuclides are applicable to the present invention and those skilled in the art are credited with the ability to readily determine which radionuclide is most appropriate under a variety of circumstances. For example, iodine [131] is a well known radionuclide used for targeted immunotherapy. However, the clinical usefulness of iodine [131] can be limited by several factors including: eight-day physical half-life; delalogenation of iodinated antibody both in the blood and at tumor sites; and emission characteristics (eg, large gamma component) which can be suboptimal for localized dose deposition in tumor. With the advent of superior chelating agents, the opportunity for attaching metal chelating groups to proteins has increased the opportunities to utilize other radionuclides such as indium [111] and yttrium [90]. Yttrium [90] provides several benefits for utilization in radioimmunotherapeutic applications: the 64 hour half-life of yttrium [90] is long enough to allow antibody accumulation by tumor and, unlike eg, iodine [131], yttrium [90] is a pure beta emitter of high energy with no accompanying gamma irradiation in its decay, with a range in tissue of 100 to 1000 cell diameters. Furthermore, the minimal amount of penetrating radiation allows for outpatient administration of yttrium [90]-labeled antibodies. Additionally, internalization of labeled antibody is not required for cell killing, and the local emission of ionizing radiation should be lethal for adjacent tumor cells lacking the target antigen.

One non-therapeutic limitation to yttrium [90] is based upon the absence of significant gamma radiation making imaging therewith difficult. To avoid this problem, a diagnostic "imaging" radionuclide, such as indium [111], can be utilized for determining the location and relative size of a tumor prior to the administration of therapeutic doses of yttrium [90]-labeled anti-CD20. Indium [111] is particularly preferred as the diagnostic radionuclide because: between about 1 to about 10 mCi can be safely administered without detectable toxicity; and the imaging data is generally predictive of subsequent yttrium [90]-labeled antibody distribution. Most imaging studies utilize 5 mCi indium [111]-labeled antibody because this dose is both safe and has increased imaging efficiency compared with lower doses, with optimal imaging occurring at three to six days after antibody administration. See, for example, Murray J. L., 26 J. Nuc. Med. 3328 (1985) and Carragiuillo, J. A. et al. 26 J. Nuc. Med. 67 (1985).

Effective single treatment dosages (ie, therapeutically effective amounts) of yttrium [90] labeled anti-CD20 antibodies range between about 5 and about 75 mCi, more preferably between about 10 and about 40 mCi. Effective single treatment non-marrow ablative dosages of iodine [131] labeled anti-CD20 antibodies range from between about 5 and about 70 mCi, more preferably between about 10 and about 40 mCi. Effective single treatment ablative dosages (ie, may require autologous bone marrow transplantation) of iodine [131] labeled anti-CD20 antibodies range from between about 30 and about 600 mCi, more preferably between about 50 and less than about 500 mCi.

In conjunction with a chimeric anti-CD20 antibody, owing to the longer circulating half-life vis-a-vis murine antibodies, an effective single treatment non-marrow ablative dosages of iodine [131] labeled chimeric anti-CD20 antibodies range from between about 5 and about 40 mCi, more preferably less than about 30 mCi. Imaging criteria for, eg, the indium [111] label, are typically less than about 5 mCi.

With respect to radiolabeled anti-CD20 antibodies, therapy therewith can also occur using a single therapy treatment or using multiple treatments. Because of the radionuclide component, it is preferred that prior to treatment, peripheral stem cells ("PSC") or bone marrow ("BM") be "harvested" for patients experiencing potentially fatal bone marrow toxicity resulting from radiation. BM and/or PSC are harvested using standard techniques, and then purged and frozen for possible reinfusion. Additionally, it is most preferred that prior to treatment a diagnostic dosimetry study using a diagnostic labeled antibody (eg, using iodine [111]) be conducted on the patient, a purpose of which is to ensure that the therapeutically labeled antibody (eg, using yttrium [90]) will not become unnecessarily "concentrated" in any normal organ or tissue.

Chimeric mouse/human antibodies have been described. See, for example, Morrison, S. L. et al., PNAS J:6851-6854 (November 1984); European Patent Publication No. 173494; Boullanne, G. L. et al., Nature 312:643 (December 1984); Neuberger, M. S. et al., Nature 314:268 (March 1985); European Patent Publication No. 125023; Tan et al., J. Immunol. 135:8564 (November 1985); Yamada K. et al., Hybridoma 5:157 (1986); Schild et al., J. Immunol. 137:1065-1074 (1986). See generally, Munro, Nature 312:597 (December 1984); Dickson, Genetic Engineering News 5/3 (March 1985); Marx, Science 229 455 (August 1985); and Morrison Science 229:1202-1207 (September 1985). Robinson et al., in PCT Publication Number WO 88/04936, describe a chimeric antibody with human constant region and murine variable region, having specificity to an epitope of CD20; the murine portion of the chimeric antibody of the Robinson references is derived from the 2H7 mouse monoclonal antibody (gamma 2b, kapp). While the reference notes that the described chimeric antibody is a "prime candidate" for the treatment of B cell disorders, this statement can be viewed as no more than a suggestion to those in the art to determine whether or not this suggestion is accurate for this particular antibody, particularly because the reference lacks any data to support an assertion of therapeutic effectiveness, and importantly, data using higher order mammals such as primates or humans.

Methodologies for generating chimeric antibodies are available to those in the art. For example, the light and heavy chains can be expressed separately, using, for example, immunoglobulin light chain and immunoglobulin heavy chains in separate plasmids. These can then be purified and assembled in vitro to complete antibodies; methodologies for accomplishing such assembly have been described. See, for example, Saffek, M., Harvey Lectures 69:125 (1975). In vitro reaction parameters for the formation of IgG antibodies from reduced isolated light and heavy chains have also been described. See, for example, Boychok, S., Cells of Immunoglobulin Synthesis. Academic Press, New York, p. 69, 1979. Co-expression of light and heavy chains in the same cells to achieve intracellular association and linkage of heavy and light chains into complete H-Lg IgG antibodies is also possible. Such co-expression can be accomplished using either the same or different plasmids in the same host cell.

Another approach, and one which is our most preferred approach for developing a chimeric non-human/human anti-
CD20 antibody, is based upon utilization of an expression vector which includes, ab initio, DNA encoding heavy and light chain constant regions from a human source. Such a vector allows for inserting DNA encoding non-human variable region such that a variety of non-human anti-CD20 antibodies can be generated, screened and analyzed for various characteristics (eg, type of binding specificity, epitope binding regions, etc); thereafter, cDNA encoding the light and heavy chain variable regions from a preferred or desired anti-CD20 antibody can be incorporated into the vector. We refer to these types of vectors as Tandem Chimera vectors ("TCAE"), including a particular TCAE vector which was used to generate immunologically active chimeric anti-CD20 antibodies for therapeutic treatment of lymphomas is TCAE 8. TCAE 8 is a derivative of a vector owned by the assignee of this patent document, referred to as TCAE 5.2 the difference being that in TCAE 5.2, the translation initiation start site of the dominant selectable marker (neomycin phosphotransferase, "NEO") is a consensus Kozak sequence, while for TCAE 8, this region is a partially impaired consensus Kozak sequence. Details regarding the impact of the initiation start sites of the dominant selectable marker of the TCAE vectors (also referred to as "ANEX vector") vis-a-vis protein expression are disclosed in detail in the co-pending application filed herewith.

TCAE 8 comprises four (4) transcriptional cassettes, and these are in tandem order, i.e., a human immunoglobulin light chain absent a variable region; a human immunoglobulin heavy chain absent a variable region; DHFR; and NEO. Each transcriptional cassette contains its own eukaryotic promoter and polyadenylation region (reference is made to FIG. I which is a diagrammatic representation of the TCAE 8 vector).

Specifically:

1) the CMV promoter/enhancer in front of the immunoglobulin heavy chain is a truncated version of the promoter/enhancer in front of the light chain, from the Herpes Simplex 1 site at -350 to the Sts 1 site at -16 (see, 41 Cell 521, 1985);

2) a human immunoglobulin light chain constant region was derived via amplification of cDNA by a PCR reaction. In TCAE 8, this was the human immunoglobulin light chain kappa constant region (Kabat numbering, amino acids 108-214, allotype Km 3, (see, Kabat, E. A. "Sequences of proteins of immunological interest." NIH Publication, Fifth Ed. No. 91-3242, 1991)); and the human immunoglobulin heavy chain gamma constant region (Kabat numbering, amino acids 114-478, allotype GmIa, Gmlz). The light chain was isolated from normal human blood (J. Immunol. (1974), 112), and sequences which maintain the translational reading frame and do not alter the amino acids normally found in immunoglobulin heavy chain constant regions, which maintain the transitional reading frame and do not alter the amino acids normally found in immunoglobulin light chain constant regions, were found in immunoglobulin heavy chain and light chain variable regions (SW). Hamster Ovary lines, DHFR minus, HELA (human cervical carcinoma), CVI (monkey kidney line), COS (a derivative of CD20 antibody, is based upon utilization of an expression vector which includes, ab initio, DNA encoding heavy and light chain constant regions from a human source. Such a vector allows for inserting DNA encoding non-human variable region such that a variety of non-human anti-CD20 antibodies can be generated, screened and analyzed for various characteristics (eg, type of binding specificity, epitope binding regions, etc); thereafter, cDNA encoding the light and heavy chain variable regions from a preferred or desired anti-CD20 antibody can be incorporated into the vector. We refer to these types of vectors as Tandem Chimera vectors ("TCAE"), including a particular TCAE vector which was used to generate immunologically active chimeric anti-CD20 antibodies for therapeutic treatment of lymphomas is TCAE 8. TCAE 8 is a derivative of a vector owned by the assignee of this patent document, referred to as TCAE 5.2 the difference being that in TCAE 5.2, the translation initiation start site of the dominant selectable marker (neomycin phosphotransferase, "NEO") is a consensus Kozak sequence, while for TCAE 8, this region is a partially impaired consensus Kozak sequence. Details regarding the impact of the initiation start site of the dominant selectable marker of the TCAE vectors (also referred to as "ANEX vector") vis-a-vis protein expression are disclosed in detail in the co-pending application filed herewith.

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Specifically:

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5) The DHFR cassette contained its own eukaryotic promoter (mouse beta globin major promoter, "BETA") and polyadenylation region (bovine growth hormone polyadenylation, "BGH"); and

6) The NEO cassette contained its own eukaryotic promoter (BETA) and polyadenylation region (SV40 early polyadenylation, "SW").

With respect to the TCAE 8 vector and the NEO cassette, the Kozak region was a partially impaired consensus Kozak sequence (SEQ. ID. NO. 1) (which included an upstream Cla I site):

TCAE 8 vector, the change is between the Clal and ATG regions, to wit: ceAatc.

The complete sequence listing of TCAE 8 (including the specific components of the four transcriptional cassettes) is set forth in FIG. 2 (SEQ. ID. NO. 2).

As will be appreciated by those in the art, the TCAE vectors beneficially allow for substantially reducing the time in generating the immunologically active chimeric anti-CD20 antibodies. Generation and isolation of non-human light and heavy chain variable regions, followed by incorporation thereof within the human light chain constant transcriptional cassette and human heavy chain constant transcriptional cassette, allows for production of immunologically active chimeric anti-CD20 antibodies.

We have derived a most preferred non-human variable region with specificity to the CD20 antigen using a murine source and hybridoma technology. Using polymerase chain reaction ("PCR") techniques, the murine light and heavy variable regions were cloned directly into the TCAE 8 vector—this is the most preferred route for incorporation of the non-human variable region into the TCAE vector. This preference is principally professed upon the efficiency of the PCR reaction and the accuracy of insertion. However, equivalent procedures for accomplishing this task are available. For example, using TCAE 8 (or an equivalent vector), the sequence of the variable region of a non-human anti-CD20 antibody can be obtained, followed by oligonucleotide synthesis of portions of the sequence or, if appropriate, the entire sequence; thereafter, the portions or the entire synthetic sequence can be inserted into the appropriate locations within the vector. Those skilled in the art are credited with the ability to accomplish this task.

Our most preferred immunologically active chimeric anti-CD20 antibodies were derived from utilization of TCAE 8 vector which included murine variable regions derived from monoclonal antibody to CD20; this antibody (to be discussed in detail infra), is referred to as "2B8." The complete sequence of the variable regions obtained from 2B8 in TCAE 8 ("anti-CD20 in TCAE 8") is set forth in FIG. 3 (SEQ. ID. NO. 3).

The host cell line utilized for protein expression is most preferably of mammalian origin; those skilled in the art are credited with ability to preferentially determine particular host cell lines which are best suited for the desired gene product to be expressed therein. Exemplary host cell lines include, but are not limited to, DG44 and DUXB11 (Chinese Hamster Ovary lines, DHFR minus), HELA (human cervical carcinoma), CVI (monkey kidney line), COS (a derivative of
CVI with SV40 T antigen), R1610 (Chinese hamster fibroblast) BALB/C3T3 (mouse fibroblast), HAK (hamster kidney line), SP2/O (mouse myeloma), P3X63-Ag8.653 (mouse myeloma), BPA-1c1BPT (bovine endothelial cells), RAJI (human lymphocyte) and 293 (human kidney). Host cell lines are typically available from commercial services, the American Tissue Culture Collection or from published literature.

Probably the host cell line is either DG44 ("CHO") or SP2/O. See Uyland, G. et al., "Effect of gamma rays and the dihydrofolate reductase locus: deletions and inversions." Som. Cell & Mol. Gen. 12/6:555--566 (1986), and Shulman, M. et al., "A better cell line for making hybridomas secreting specific antibodies." Nature 276:269 (1978), respectively. Most preferably, the host cell line is DG44. Transfection of the plasmid into the host cell can be accomplished by any technique available to those in the art. These include, but are not limited to, transfection (including electroporation and electroporation), cell fusion with enveloped DNA, microinjection, and infection with intact virus. See, Ridgway, A. A. G., "Mammalian Expression Vectors," Chapter 24.2, pp. 470--472 Vectors, Rodriguez and Denhardt, Eds. (Butterworths, Boston, Mass. 1988). Most preferably, plasmid introduction into the host cell is via electroporation.

F. EXAMPLES

The following examples are not intended, nor are they to be construed, as limiting the invention. The examples are intended to evidence: dose-imaging using a radiolaabeled anti-CD20 antibody ("ZB2"); radiolaabeled anti-CD20 antibody ("Y2B8"); and immunologically active, chimeric anti-CD20 antibody ("Y2B8") derived utilizing a specific vector ("TCAE") and variable regions derived from murine anti-CD20 monoclonal antibody ("ZB2").

I. RADIONLABLED ANTI-CD20 ANTIBODY 2B8

A. Anti-CD20 Monoclonal Antibody (Murine) Production ("ZB2")

BALB/C mice were repeatedly immunized with the human lymphoblastic cell line SB (see, Adams, R. A. et al., "Direct implantation and serial transplantation of human acute lymphoblastic leukemia in hamsters, SB-2." Can Res 28:1121--1125 (1968); this cell line is available from the American Tissue Culture Collection, Rockville, Md., under ATCC accession number ATCC CCL 120), with weekly injections over a period of 3--4 months. Mice evidencing high serum titers of anti-CD20 antibodies, as determined by inhibition of known CD20-specific antibodies (anti-CD20 antibodies utilized were Leu 16, Beckton Dickinson, San Jose, Calif., Cat. No. 7670; and B1, Coulter Corp., Hialeah, Fla., Cat. No. 6602021) were identified; the spleens of such mice were then removed. Spleen cells were fused with the mouse myeloma SP2/O in accordance with the protocol described in Einfeld, D. A. et al., (1988) EMBO 7:711 (SP2/O has ATCC accession no. ATCC CRL 8006).

Assays for CD20 specificity were accomplished by radioimmunoassay. Briefly, purified anti-CD20 B1 was radiolaabeled with 125I by the iodode method as described in Valentine, M. A. et al., (1989) J. Biol. Chem. 264:11282. (125I Sodium Iodide, ICN, Irvine, Calif., Cat. No. 28605B). Hybridomas were screened by co-incubation of 0.05 ml of media from each of the fusion wells together with 0.05 ml of 125I labeled anti-CD20 B1 (10 ng) in 1% BSA, PBS (pH 7.4), and 0.5 ml of the same buffer containing 100,000 SB cells. After incubation for 1 hr at room temperature, the cells were harvested by transferring to 96 well titer plates (V&P Scientific, San Diego, Calif.), and washed thoroughly. Duplicate wells containing unlabeled anti-CD20 B1 and wells containing no inhibiting antibody were used as positive and negative controls, respectively. Wells containing greater than 50% inhibition were expanded and cloned. The antibody demonstrating the highest inhibition was derived from the cloned cell line designated herein as "2B8."

B. Preparation of 2B8-MX-DTPA Conjugate

I. MX-DTPA

Carbon-14-labeled 1-iodothyocyanato-benzyl-3-methylidene triaminopentanoic acid ("carbon-14 labeled MX-DTPA") was used as a chelating agent for conjugation of radiolabel to 2B8. Manipulations of MX-DTPA were conducted to maintain metal-free conditions, i.e., metal-free reagents were utilized and, when possible, polypropylene plastic containers (flasks, beakers, graduated cylinders, pipette tips) washed with ALCONOX® (a detergent) and rinsed with MILLI-Q water (purified water), were similarly utilized. MX-DTPA was obtained as a dry solid from Dr. Otto Gansow (National Institute of Health, Bethesda, Md.) and stored desiccated at 4°C (protected from light), with stock solutions being prepared in MILLI-Q® water at a concentration of 2--5 mM, with storage at 70°C. MX-DTPA was also obtained from Couler Immunology (Hialeah, Fla.) as the disodium salt in water and stored at 70°C.

ii. Preparation of 2B8

Purified 2B8 was prepared for conjugation with MX-DTPA by transferring the antibody into metal-free 50 mM bicine-NaOFF, pH 8.6, containing 150 mM NaCl, using repetitive buffer exchange with CENTRICON 30™ spin filters (30,000D, MWCO; Amicon). Generally, 50--200 μL of protein (10 mg/ml) was added to the filter unit, followed by 2 ml of bicine buffer. The filter was centrifuged at 4°C in a Sorval SS-34 rotor (6000 rpm, 45 min.). Retentate volume was approximately 50--100 μL; this process was repeated twice using the same filter. Retentate was transferred to a polypropylene 1.5 mL screw cap tube, assayed for protein, diluted to 10.0 mg/mL and stored at 4°C until utilized; protein was similarly transferred into 50 mM sodium citrate, pH 5.5, containing 150 mM NaCl and 0.05% sodium azide, using the foregoing protocol.

iii. Conjugation of 2B8 with MX-DTPA

Conjugation of 2B8 with MX-DTPA was performed in polypropylene tubes at ambient temperature. Frozen MX-DTPA stock solutions were thawed immediately prior to use, 50--200 ml of protein at 10 mg/mL were reacted with MX-DTPA at a molar ratio of MX-DTPA-to-2B8 of 4:1. Reactions were initiated by adding the MX-DTPA stock solution and gently mixing; the conjugation was allowed to proceed overnight (14 to 20 hr.), at ambient temperature. Unreacted MX-DTPA was removed from the conjugate by dialysis or repetitive ultrafiltration, as described above in Example 1B.ii. into metal-free normal saline (0.9% w/v) containing 0.05% sodium azide. The protein concentration was adjusted to 10 mg/mL and stored at 4°C in a polypropylene tube until radiolabeled.

iv. Determination of MX-DTPA Incorporation

MX-DTPA incorporation was determined by scintillation counting and comparing the value obtained with the purified antibody conjugate to the specific activity of the carbon-14-labeled MX-DTPA. For certain studies, in which non-radioactive MX-DTPA (Couler Immunology) was utilized, MX-DTPA incorporation was assessed by incubating the conjugate with an excess of a radioactive carrier solution of yttrium-[90] of known concentration and specific activity.

A stock solution of yttrium chloride of known concentration was prepared in metal-free 0.05N HCl to which carrier-free yttrium-[90] (chloride salt) was added. An aliquot of
this solution was analyzed by liquid scintillation counting to determine an accurate specific activity for this reagent. A volume of the yttrium chloride reagent equal to 3-4 times the number of moles of chelate expected to be attached to the antibody, (typically 2 mol/mol antibody), was added to a polypropylene tube, and the pH adjusted to 4.0–4.4 with 2M sodium acetate. Conjugated antibody was subsequently added and the mixture incubated 15–30 min. at ambient temperature. The reaction was quenched by adding 20 mM EDTA to a final concentration of 1 mM and the pH of the solution adjusted to approximately pH 6 with 2M sodium acetate.

After a 5 min. incubation, the entire volume was purified by high-performance, size-exclusion chromatography (described infra). The eluted protein-containing fractions were combined, the protein concentration determined, and an aliquot assayed for radioactivity. The chelate incorporation was calculated using the specific activity of the yttrium-[90] chloride preparation and the protein concentration.

v. Immunoactivity of 2B8-MX-DTPA
The immunoactivity of conjugated 2B8 was assessed using whole-cell ELISA. Mid-log phase SB cells were harvested from culture by centrifugation and washed two times with 1× HBSS. Cells were diluted to 1–2×10^6 cells/mL in HBSS and aliquoted into 96-well polystyrene microtiter plates at 50,000–100,000 cells/well. The plates were dried under vacuum for 2 h. at 40°–45° C. to fix the cells to the plastic; plates were stored dry at 20° C. until utilized. For assay, the plates were warmed to ambient temperature immediately before use, then blocked with 1× PBS, pH 7.2–7.4 containing 1% BSA (2 h). Samples for assay were diluted in 1× PBS/1% BSA, applied to plates and serially diluted 1:2 into the same buffer. After incubating plates for 1 h. at ambient temperature, the plates were washed three times with 1× PBS. Secondary antibody (goat anti-mouse IgGl-specific HRP conjugate 50 μL) was added to wells (1:1500 dilution in 1× PBS/1% BSA) and incubated 1 h. at ambient temperature. Plates were washed four times with 1× PBS followed by the addition of ABTS substrate solution (50 mM sodium citrate, pH 4.5 containing 0.01% ABTS and 0.001% H₂O₂). Plates were read at 405 nm after 15–30 min. incubation. Antigen-negative HSB cells were included in assays to monitor non-specific binding. Immunoactivity of the conjugate was calculated by plotting the absorbance values vs. the respective dilution factor and comparing these to values obtained using native antibody (representing 100% immunoactivity) tested on the same plate; several values on the linear portion of the titration profile were compared and a mean value determined (data not shown).

vi. Preparation of Indium-[111]-Labeled 2B8-MX-DTPA
(“2B88”)
Conjugates were radiolabeled with carrier-free indium-[111]. An aliquot of isotope (0.1–2 mCi/mg antibody) in 0.05M HCl was transferred to a polypropylene tube and approximately one-tenth volume of metal-free 2M HCl added. After incubation for 5 min., metal-free 2M sodium acetate was added to adjust the solution to pH 4.0–4.4. Approximately 0.5 mg of 2B8-MX-DTPA was added from a stock solution of 10.0 mg/mL DTPA in normal saline, or 50 mM sodium citrate/150 mM NaCl containing 0.05% sodium azide, and the solution gently mixed immediately. The pH solution was checked with pH paper to verify a value of 4.0–4.5 and the mixture incubated at ambient temperature for 15–30 min. Subsequently, the reaction was quenched by adding 20 mM EDTA to a final concentration of 1 mM and the reaction mixture was adjusted to approximately pH 6.0 using 2M sodium acetate.

After a 5–10 min. incubation, uncomplexed radioisotope was removed by size-exclusion chromatography. The HPLC unit consisted of Waters Model 6000 or Tosohaas Model TSK-6110 solvent delivery system fitted, respectively, with a Waters U6K or Rheodyne 700 injection valve. Chromatographic separations were performed using a gel permeation column (BioRad SEC-250; 7.5×300 mm or comparable Tosohaas column) and a SEC-250 guard column (7.5×100 mm). The system was equipped with a fraction collector (Pharmacia Fracon) and a UV monitor fitted with a 280 nm filter (Pharmacia model UV-1). Samples were applied and eluted isocratically using 1× PBS, pH 7.4, at 1.0 mL/min flow rate. One-half milliliter fractions were collected in glass tubes and aliquots of these counted in a gamma counter. The lower and upper windows were set to 100 and 500 KeV respectively.

The radioimmunoassay was calculated by summing the radioactivity associated with the eluted protein peak and dividing this number by the total radioactivity eluted from the column; this value was then expressed as a percentage (data not shown). In some cases, the radioimmunoassay was determined using instant thin-layer chromatography (“ITLC”). Radio-labeled conjugate was diluted 1:10 or 1:20 in 1× PBS containing or 1× PBS/1 mM DTPA, then 1 mL was spotted 1.5 cm from one end of a 2×5 cm strip of ITLC SG paper. The paper was developed by ascending chromatography using 10% ammonium acetate in methanol:water (1:1; v/v). The strip was dried, cut in half crosswise, and the radioactivity associated with each section determined by gamma counting. The radioactivity associated with the bottom half of the strip (protein-associated radioactivity) was expressed as a percentage of the total radioactivity, determined by summing the values for both top and bottom halves (data not shown).

Specific activities were determined by measuring the radioactivity of an appropriate aliquot of the radiolabeled conjugate. This value was corrected for the counter efficiency (typically 75%) and related to the protein concentration of the conjugate, previously determined by absorbance at 280 nm, and the resulting value expressed as mCi/mg protein.

For some experiments, 2B8-MX-DTPA was radiolabeled with indium [111] following a protocol similar to the one described above but without purification by HPLC; this was referred to as the “mix-and-shoot” protocol.

vii. Preparation of Yttrium-[90]-Labeled 2B8-MX-DTPA ("Y2B8")
The same protocol described for the preparation of 2B8 was followed for the preparation of the yttrium-[90]-labeled 2B8-MX-DTPA ("Y2B8") conjugate except that 2 ng HCl was not utilized; all preparations of yttrium-labeled conjugates were purified by size-exclusion chromatography as described above.

C. Non-Human Animal Studies
i. Biodistribution of Radiolabeled 2B8-MX-DTPA
2B8 was evaluated for tissue biodistribution in six to eight week old BALB/c mice. The radiolabeled conjugate was prepared using clinical-grade 2B8-MX-DTPA following the "mix and shoot" protocol described above. The specific activity of the conjugate was 2.3 mCi/mg and the conjugate was formulated in PBS, pH 7.4 containing 50 mg/mL HSA. Mice were injected intravenously with 100 μL of 2B8 (approximately 21 μCi) and groups of three mice were sacrificed by cervical dislocation at 0, 24, 48, and 72 hours. After sacrifice, the tail, heart, lungs, liver, kidney, spleen, muscle, and femur were removed, washed and weighed; a sample of blood was also removed for analysis.
Radioactivity associated with each specimen was determined by gamma counting and the percent injected dose per gram tissue subsequently determined. No attempt was made to discount the activity contribution represented by the blood associated with individual organs.

In a separate protocol, aliquots of 2B8-MX-DTPA incubated at 4°C and 30°C for 10 weeks were radiolabeled with indium-[111] to a specific activity of 2.3 mCi/mg for both preparations. These conjugates were then used in biodistribution studies in mice as described above.

For dosimetry determinations, 2B8-MX-DTPA was radiolabeled with indium-[111] to a specific activity of 2.3 mCi/mg and approximately 1 μCi was injected into each of 20 BALB/c mice. Subsequently, groups of five mice each were sacrificed at 1, 24, 48 and 72 hours and their organs and tissues analyzed for activity. The organs, portions of the skin, muscle, and bone were removed and processed for analysis. The urine and feces were also collected and analyzed for the 24–72 hour time points.

Using a similar approach, 2B8-MX-DTPA was also radiolabeled with yttrium-[90] and its biodistribution evaluated in BALB/c mice over a 72-hour time period. Following purification by HPLC size exclusion chromatography, four groups of five mice each were injected intravenously with approximately 1 μCi of clinically-formulated conjugate (specific activity: 12.2 mCi/mg); groups were subsequently sacrificed at 1, 24, 48 and 72 hours and their organs and tissues analyzed as described above. Radioactivity associated with each tissue specimen was determined by measuring bremsstrahlung energy with a gamma scintillation counter. Activity values were subsequently expressed as percent injected dose per gram tissue or percent injected dose per organ. While organs and other tissues were rinsed repeatedly to remove superficial blood, the organs were not perfused. Thus, organ activity values were not discounted for the activity contribution represented by internally associated blood.

ii. Tumor Localization of 2B8

The localization of radiolabeled 2B8-MX-DTPA was determined in athymic mice bearing Ramos B cell tumors. Six- to eight-week-old athymic mice were injected subcutaneously (left rear flank) with 0.1 mL of RPMI-1640 containing 1.2×10^7 Ramos tumor cells which had been previously adapted for growth in athymic mice. Tumors arose within two weeks and ranged in weight from 0.07 to 1.1 grams. Tumors were injected intravenously with 100 μL of indium-[111]-labeled 2B8-MX-DTPA (16.7 μCi) and groups of three mice were sacrificed by cervical dislocation at 0, 24, 48, and 72 hours. After sacrifice the tail, heart, lungs, liver, kidney, spleen, muscle, femur, and tumor were removed, washed, weighed; a sample of blood was also removed for analysis. Radioactivity associated with each specimen was determined by gamma counting and the percent injected dose per gram tissue determined.

iii. Biodistribution and Tumor Localization Studies with Radiolabeled 2B8-MX-DTPA

Following the preliminary biodistribution experiment described above (Example I.B. viii.), conjugated 2B8 was radiolabeled with indium-[111] to a specific activity of 2.3 mCi/mg and roughly 1.1 μCi was injected into each of twenty BALB/c mice to determine biodistribution of the radiolabeled material. Subsequently, groups of five mice each were sacrificed at 1, 24, 48 and 72 hours and their organs and a portion of the skin, muscle and bone were removed and processed for analysis. In addition, the urine and feces were collected and analyzed for the 24–72 hour time points. The level of radioactivity in the blood dropped from 40.3% of the injected dose per gram at 1 hour to 18.9% at 72 hours (data not shown). Values for the heart, kidney, muscle and spleen remained in the range of 0.7–9.8% throughout the experiment. Levels of radioactivity found in the lungs decreased from 14.2% at 1 hour to 7.6% at 72 hours; similarly, the respective liver injected-dose per gram values were 10.3% and 9.0%. These data were used in determining radiation absorbed dose estimates for Yb8 described below.

The biodistribution of yttrium-[90]-labeled conjugate, having a specific activity of 12.2 mCi/mg antibody, was evaluated in BALB/c mice. Radioinfectious conjugates of >90% were obtained and the radiolabeled antibody was purified by HPLC. Tissue deposition of radioactivity was evaluated in the major organs, the skin, muscle, bone, and skin and feces over 72 hours and expressed as percent injected dose/g tissue. Results (not shown) evidenced that while the levels of radioactivity associated with the blood dropped from approximately 39.2% injected dose per gram at 1 hour to roughly 15.4% after 72 hours the levels of radioactivity associated with the bone ranged from 4.4% of the injected dose per gram bone at 1 hour to 1.5% at 72 hours. Taken together, these results suggest that little free yttrium was associated with the conjugate and that little free radiometric was released during the course of the study. These data were used in determining radiation absorbed dose estimates for Yb8 described below.

For tumor localization studies, 2B8-MX-DTPA was prepared and radiolabeled with 111-In. Indium to a specific activity of 2.7 mCi/mg. One hundred microliters of labeled conjugate (approximately 24 μCi) were subsequently injected into each of 12 athymic mice bearing Ramos B cell tumors. Tumors ranged in weight from 0.1 to 1.0 grams. At time points of 0, 24, 48, and 72 hours following injection, 50 μL of blood was removed by retro-orbital puncture, the mice sacrificed by cervical dislocation, and the tail, heart, lungs, liver, kidney, spleen, muscle, femur, and tumor removed. After processing and weighing the tissues, the radioactivity associated with each tissue specimen was determined using a gamma counter and the values expressed as percent injected dose per gram.

The results (not shown) evidenced that the tumor concentrations of the 111-In-2B8-MX-DTPA increased steadily throughout the course of the experiment. Thirteen percent of the injected dose was accumulated in the tumor after 72 hours. The blood levels, by contrast, dropped during the experiment from over 30% at time zero to 13% at 72 hours. All other tissues (except muscle) contained between 1.3 and 6.0% of the injected dose per gram tissue by the end of the experiment; muscle tissue contained approximately 13% of the injected dose per gram.

D. Human Studies

1. 2B8 and 2B8-MX-DTPA: Immunohistochemistry Studies with Human Tissues

The tissue reactivity of murine monoclonal antibody 2B8 was evaluated using a panel of 32 different human tissues fixed with acetone. Antibody 2B8 reacts with the anti-CD20 antigen which had a very restricted pattern of tissue distribution, being observed only in a subset of cells in lymphoid tissues including those of hematopoietic origin.

In the lymph node, immunoreactivity was observed in a population of mature cortical B-lymphocytes as well as proliferating cells in the germinal centers. Positive reactivity was also observed in the peripheral blood, B-cell areas of the...
tonsils, white pulp of the spleen, and with 40-70% of the medullary lymphocytes found in the thymus. Positive reactivity was also seen in the follicles of the lamina propria (Peyer’s Patches) of the large intestines. Finally, aggregates or scattered lymphoid cells in the stroma of various organs, including the bladder, breast, cervix, esophagus, lung, parotid, prostate, small intestine, and stomach, were also positive with antibody 2B8 (data not shown).

All simple epithelial cells, as well as the stratified epithelium and epithelia of different organs, were found to be unreactive. Similarly, no reactivity was seen with neuroectodermal cells, including those in the brain, spinal cord and peripheral nerves, Mesenchymal elements, such as skeletal and smooth muscle cells, fibroblasts, endothelial cells, and polymorphonuclear inflammatory cells were also found to be negative (data not shown).

The tissue reactivity of the 2B8-MX-DTPA conjugate was evaluated using a panel of sixteen human tissues which had been fixed with acetone. As previously demonstrated with the native antibody (data not shown), the 2B8-MX-DTPA conjugate recognized the CD20 antigen which exhibited a highly restricted pattern of distribution, being found only on a subset of cells of lymphoid origin. In the lymph node, immunoreactivity was observed in the B cell population. Strong reactivity was seen in the white pulp of the spleen and in the medullary lymphocytes of the thymus. Immunoreactivity was also observed in scattered lymphocytes in the bladder, heart, large intestines, liver, lung, and uterus, and was attributed to the presence of inflammatory cells present in these tissues. As with the native antibody, no reactivity was observed with neuroectodermal cells or with mesenchymal elements (data not shown).

ii. Clinical Analysis of 2B8 (Imaging) and 2YB8 (Therapy)

a. Phase I/II Clinical Trial Single Dose Therapy Study

A Phase I/II clinical analysis of 2B8 (imaging) followed by treatment with a single therapeutic dose of 2YB8 is currently being conducted. For the single-dose study, the following schema is being followed:

1. Peripheral Stem Cell (PSC) or Bone Marrow (BM) Harvest with Perfusion
2. 2B8 Imaging;
3. 2YB8 Therapy (three Dose Levels); and
4. PSC or Autologous BM Transplantation (based upon decision of Medical practitioner).

The Dose Levels of 2YB8 are as follows:

<table>
<thead>
<tr>
<th>Dose Level</th>
<th>Dose (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>10</td>
</tr>
<tr>
<td>2.</td>
<td>15</td>
</tr>
<tr>
<td>3.</td>
<td>20</td>
</tr>
</tbody>
</table>

Three patients are to be treated at each of the dose levels for determination of an MTD.

Imaging (Dosimetry) Studies are conducted as follows: A preferred imaging dose for the unlabeled antibody (2B8) will be determined with the first two patients. The first two patients will receive 100 mg of unlabeled 2B8 in 250 cc of normal saline over 4 hours followed by 0.5 mCi of 2B8—blood will be sampled for biodistribution data at times t=0, t=10 min., t=120 min., t=24 hr, and t=48 hr. Patients will be scanned with multiple regional gamma camera images at times t=2 hr, t=24 hr and t=48 hr. After scanning at t=48 hr, the patients will receive 250 mg of 2B8 as described, followed by 4.5 mCi of 2B8—blood and scanning will then follow as described. If 100 mg of 2B8 produces superior imaging, then the next two patients will receive 50 mg of 2B8 as described, followed by 0.5 mCi of 2B8—blood and then with 4.5 mCi of 2B8. If 250 mg of 2B8 produces superior imaging, then the next two patients will receive 250 mg of 2B8 as described, followed by 0.5 mCi of 2B8—blood and then with 4.5 mCi of 2B8. Subsequent patients will be treated with the lowest amount of 2B8 that provides optimal imaging. Optimal imaging will be defined by: (1) best effective imaging with the slowest disappearance of antibody; (2) best distribution minimizing compartmentalization in a single organ; and (3) best subjective resolution of the lesion (tumor/background comparison).
For the first four patients, the first therapeutic dose of Y2B8 will begin 14 days after the last dose of I2B8; for subsequent patients, the first therapeutic dose of Y2B8 will begin between two to seven days after the I2B8.

Prior to treatment with Y2B8, for the patients other than the first four, 2B8 will be administered as described, followed by i.v. infusion of Y2B8 over 5–10 min. Blood will be sampled for biodistribution at times t=0, t=10 min., t=120 min., t=24 hr and t=48 hr. Patients will receive repetitive doses of Y2B8 (the same dose administered as with the first dose) approximately every six to eight weeks for a maximum of four doses, or total cumulative dose of 80 mg. It is most preferred that patients not receive a subsequent dose of Y2B8 until the patients' WBC is greater than/equal to 3,000 and AQC is greater than/equal to 100,000.

Following completion of the three-dose level study, an MTD will be defined. Additional patients will then be enrolled in the study and these will receive the MTD.

II. CHIMERIC ANTI-CD20 ANTIBODY PRODUCTION (“CB20”)

Construction of Chimeric Anti-CD20 Immunoglobulin DNA Expression Vector

DNA was isolated from the 2B8 mouse hybridoma cell (as described in Chomczynski, P. et al., “Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-
chloroform extraction.” Anak Biochem. 162:150–159 (1987)). Extract was prepared therefrom. The mouse immunoglobulin light chain variable region DNA was isolated from the cDNA by polymerase chain reaction using a set of DNA primers with homology to mouse light chain signal sequences at the 5’ end and mouse light chain J region at the 3’ end. Primer sequences were as follows:

1. V\(_{\text{H}}\) Sense (SEQ. ID. NO. 4)

\[
\text{AGATCATCTGGGTACGGGGTTTCTACGAGCAGCTCAG}
\]

2. V\(_{\text{L}}\) Antisense (SEQ. ID. NO. 5)

\[
\text{TACACGGCAGCCGGCGTTCTACGAGCAGCTCAG}
\]

The underlined portion is a Bgl II site; the above-lined portion is the start codon.

These resulting DNA fragments were cloned directly into the TCAE 8 vector in front of the human kappa light chain constant domain and sequenced. The determined DNA sequence for the murine variable region light chain is set forth in FIG. 4 (SEQ. ID. NO. 6); see also FIGS. 3A–F (SEQ. ID. NO. 3) for the corresponding sites in anti-CD20 in TCAE 8.

These resulting DNA fragments were cloned directly into the TCAE 8 vector in front of the human kappa light chain constant domain, sequenced. The determined DNA sequence for the murine variable region light chain is set forth in FIG. 4 (SEQ. ID. NO. 6); see also FIGS. 3A–F (SEQ. ID. NO. 3), nucleotides 978 through 1362. FIG. 4 further provides the amino acid sequence from this murine variable region, and the CDR and framework regions. The mouse light chain variable region from 2B8 is in the mouse kappa VI family. See, Kabat, supra.

The mouse heavy chain variable region was similarly isolated and cloned in front of the human IgG1 constant domains. Primers were as follows:

1. V\(_{\text{H}}\) Sense (SEQ. ID. NO. 7)

\[
\text{GACGGCTTCCACCCTGCGTTCTCACGAGCTCAG}
\]

2. V\(_{\text{L}}\) Antisense (SEQ. ID. NO. 8)

\[
\text{GATGAAGACAATACCTGGGAAGCTGATGGAATGATG}
\]

The underlined portion is an Mlu I site.

The sequence for this mouse heavy chain is set forth in FIG. 5 (SEQ. ID. NO. 9); see also FIGS. 3A–F (SEQ. ID. NO. 3), nucleotide 2401 through 2820. FIG. 5 also provides the amino acid sequence from this murine variable region, and the CDR and framework regions. The mouse heavy chain variable region from 2B8 is in the mouse VH 2B family. See, Kabat, supra.

B. Creation of Chimeric Anti-CD20 Producing CHO and SP2/0 Transfectomas

Chinese hamster ovary (“CHO”) cells DG44 were grown in SSFM II minus hypoxanthine and thymidine media (Gibco, Grand Island, N.Y. Form No. 91-045466); SP2/0 mouse myeloma cells were grown in Dulbecco’s Modified Eagles Medium media (“DMEM”) (Irvine Scientific, Santa Ana, Calif., Cat. No. 9024) with 5% fetal bovine serum and 20 ml/L glutamine added. Four million cells were electroporated with either 25 μg CHO or 50 μg SP2/0 plasmid DNA that had been restricted with Not I using a BTX 600 electroporation system (BTX, San Diego, Calif.) in 0.4 ml disposable cuvettes. Conditions were either 210 volts for CHO or 180 volts for SP2/0, 400 microfaradays. 13 ohms. Each electroporation was plated into six 96 well dishes (about 7,000 cells/well). Dishes were fed with media containing G418 (GENETICIN, Gibco, Cat. No. 860-1811) at 400 μg/ml active compound for CHO (media further included 50 μM hypoxanthine and 8 μM thymidine) or 800 μg/ml for SP2/0, two days following electroporation and thereafter 2 or 3 days until colonies arose. Supernatant from colonies was assayed for the presence of chimeric immunoglobulin via an ELISA specific for human antibody. Colonies producing the highest amount of immunoglobulin were expanded and plated into 96 well plates containing media plus methotrexate (25 nM for SP2/0 and 5 nM for CHO) and fed every two or three days. Supernatants were assayed as above and colonies producing the highest amount of immunoglobulin were examined. Chimeric anti-CD20 antibody was purified from supernatant using protein A affinity chromatography.

Purified chimeric anti-CD20 was analyzed by electrophoresis in polyacrylamide gels and estimated to be greater than about 95% pure. Affinity and specificity of the chimeric antibody was determined based upon 2B8. Chimeric anti-CD20 antibody tested in direct and competitive binding assays, when compared to murine anti-CD20 monoclonal antibody 2B8, evidenced comparable affinity and specificity on a number of CD20 positive B cells lines (data not presented). The apparent affinity constant (“Kap”) of the chimeric antibody was determined by direct binding of 125I radiolabeled chimeric anti-CD20 and compared to radiola
dered 2B8 by Scatchard plot; estimated Kap for CHO produced chimeric anti-CD20 was 5.2×10^{-10} M and for SP2/0 produced antibody, 7.4×10^{-10} M. The estimated Kap for 2B8 was 3.5×10^{-9} M. Direct competition by radiolimmunoassay was utilized to confirm both the specificity and retention of immunoreactivity of the chimeric antibody by comparing its ability to effectively compete with 2B8. Substantially equivalent amounts of chimeric anti-CD20 and 2B8 antibodies were required to produce 50% inhibition of binding to CD20 antigens on B cells (data not presented), i.e., there

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was a minimal loss of inhibiting activity of the anti-CD20 antibodies, presumably due to chimerization. The results of Example IIB indicate, inter alia, that chimeric anti-CD20 antibodies were generated from CHO and SP2/0 transfectedomas using the TCAE 8 vectors, and these chimeric antibodies had substantially the same specificity and binding capability as murine anti-CD20 monoclonal antibody 2B8.

C. Determination of Immunological Activity of Chimeric Anti-CD20 Antibodies

i. Human C1q Analysis

Chimeric anti-CD20 antibodies produced by both CHO and SP2/0 cell lines were evaluated for human C1q binding in a flow cytometry assay using fluorescein labeled C1q (C1q was obtained from Quidel, Mira Mesa, Calif., Prod. No. A400 and FITC label from Sigma, St. Louis Mo., Prod. No. F-7250; FITC. Labeling of C1q was accomplished in accordance with the protocol described in Selected Methods In Cellular Immunology, Michell & Shulgi, Ed. (W. H. Freeman & Co., San Francisco, Calif., 1983, p. 292). Analytical results were derived using a Becton Dickinson FACScan™ flow cytometer (fluorescein measured over a range of 515–545 nm). Equivalent amounts of chimeric anti-CD20 antibody, human IgG1.K myeloma protein (Binding Site, San Diego, Calif., Prod. No. BP078), and 2B8 were incubated with an equivalent number of CD20-positive SB cells, followed by a wash step with FACS buffer (0.2% BSA in PBS, pH 7.4, 0.02% sodium azide) to remove unattached antibody, followed by incubation with FITC labeled C1q. Following a 30–60 min. incubation, cells were again washed. The three conditions, including FITC-labeled C1q as a control, were analyzed on the FACScan™ following manufacturing instructions. Results are presented in FIG. 6.

As the results of FIG. 6 evidence, a significant increase in fluorescence was observed only for the chimeric anti-CD20 antibody condition; i.e., only SB cells with adherent chimeric anti-CD20 antibody were C1q positive, while the other conditions produced the same pattern as the control.

ii. Complement Dependent Cell Lysis

Chimeric anti-CD20 antibodies were analyzed for their ability to lyse lymphoma cell lines in the presence of human serum (complement source). CD20 positive SB cells were labeled with 51Cr by admixing 100 μCi of 51Cr with 1×10⁶ SB cells for 1 hr at 37°C; labeled SB cells were then incubated in the presence of equivalent amounts of human complement and equivalent amounts of 0–50 μg/ml of either chimeric anti-CD20 antibodies or 2B8 for 4 hrs at 37°C. (see, Brunnner, K. T. et al., “Quantitative assay of the lytic action of immune lymphoid cells on 51Cr-labeled allogeneic target cells in vitro.” Immunology 14:181–189 (1968). Results are presented in FIG. 7.

The results of FIG. 7 indicate, inter alia, that chimeric anti-CD20 antibodies produced significant lysis (49%) under these conditions.

iii. Antibody Dependent Cellular Cytotoxicity Effector Assay

For this study, CD20 positive cells (SB) and CD20 negative cells (T cell leukemia line HSB; see, Adams, Richard, “Formal Discussion,” Can. Res. 27:2479–2482 (1967); ATCC deposit no. ATCC CCL 120.1) were utilized; both were labeled with 51Cr. Analysis was conducted following the protocol described in Brunn, K. T. et al., “Quantitative assay of the lytic action of immune lymphoid cells on 51Cr-labeled allogeneic target cells in vitro; inhibition by isonitro and drugs.” Immunology 14:181–189 (1968); a substantial chimeric anti-CD20 antibody dependent cell mediated lysis of CD20 positive SB target cells (51Cr-labeled) at the end of a 4 hr, 37°C incubation, was observed and this effect was observed for both CHO and SP2/0 produced antibody (effector cells were human peripheral lymphocytes; ratio of effector cell:target was 100:1). Efficient lysis of target cells was obtained at 3.9 μg/ml. In contrast, under the same conditions, the murine anti-CD20 monoclonal antibody 2B8 had a statistically insignificant effect, and CD20 negative HSB cells were not lysed. Results are presented in FIG. 8.

The results of Example II indicate, inter alia, that the chimeric anti-CD20 antibodies of Example I were immunologically active.

III. DEPLETION OF B CELLS IN VIVO USING CHIMERIC ANTI-CD20

A. Non-Human Primate Study

Three separate non-human primate studies were conducted. For convenience, these are referred to herein as “Chimeric Anti-CD20: CHO & SP2/0,” “Chimeric Anti-CD20: CHO,” and “High Dose Chimeric Anti-CD20.” Conditions were as follows:

Chimeric Anti-CD20: CHO & SP2/0

Six cynomolagus monkeys ranging in weight from 4.5 to 7 kilograms (White Sands Research Center, Alamogordo, N. Mex.) were divided into three groups of two monkeys each. Both animals of each group received the same dose of immunologically active chimeric anti-CD20 antibody. One animal in each group received purified antibody produced by the CHO transfectedoma; the other received antibody produced by the SP2/0 transfectedoma. The three groups received antibody dosages corresponding to 0.1 mg/kg, 0.4 mg/kg, and 1.6 mg/kg each day for four (4) consecutive days.

Chimeric immunologically active anti-CD20 antibody, which was admixed with sterile saline, was administered by intravenous infusion; blood samples were drawn prior to each infusion. Additional blood samples were drawn beginning 24 hrs after the last injection (T=0) and thereafter on days 1, 3, 7, 14, and 28; blood samples were also taken thereafter at biweekly intervals until completion of the study at day 90.

Approximately 5 ml of whole blood from each animal was centrifuged at 2000 RPM for 5 min. Plasma was removed for assay of soluble chimeric anti-CD20 antibody levels. The pellet (containing peripheral blood leukocytes and red blood cells) was resuspended in fetal calf serum for fluorescent-labeled antibody analysis (see, “Fluorescent Antibody Labeling of Lymphoid Cell Population,” infra.).

Chimeric Anti-CD20: CHO

Six cynomolagus monkeys ranging in weight from 4 to 6 kilograms (White Sands) were divided into three groups of two monkeys each. All animals were injected with immunologically active chimeric anti-CD20 antibodies produced from the CHO transfectedoma (in sterile saline). The three groups were separated as follows: subgroup 1 received daily intravenous injections of 0.01 mg/kg of the antibody over a four (4) day period; subgroup 2 received daily intravenous injections of 0.4 mg/kg of the antibody over a four (4) day period; subgroup 3 received a single intravenous injection of 6.4 mg/kg of the antibody. For all three subgroups, a blood sample was obtained prior to initiation of treatment; additionally, blood samples were also drawn at T=0, 1, 3, 7, 14 and 28 days following the last injection, as described above, and these samples were processed for fluorescent labeled antibody analysis (see, “Fluorescent Antibody Labeling,” infra.). In addition to peripheral blood B cell quantitation, lymph node biopsies were taken at days 7, 14 and 28 following the last injection, and a single cell preparation stained for quantitation of lymphocyte populations by flow cytometry.
High Dosage Chimeric Anti-CD20

Two cynomolgus monkeys (White Sands) were infused with 16.8 mg/kg of the immunologically active chimeric anti-CD20 antibodies from the CHO transfectomas (in sterile saline) weekly over a period of four consecutive weeks. At the conclusion of the treatment, both animals were anesthetized for removal of bone marrow; lymph node biopsies were also taken. Both sets of tissue were stained for the presence of B lymphocytes using Leu 16 by flow cytometry following the protocol described in Ling, N. R. et al., "B-cell and plasma cell antigens." Leucocyte Typing III White Cell Differentiations Antigens, A. J. McMichael, Ed. (Oxford University Press, Oxford UK, 1987). p. 302.

Fluorescent Antibody Labeling of Lymphoid Cell Population

After removal of plasma, leukocytes were washed twice with Hank's Balanced Salt Solution ("HBSS") and resuspended in a plasma equivalent volume of fetal bovine serum (heat inactivated at 56°C for 30 min.). A 0.1 ml volume of the cell preparation was distributed to each of six (6), 15 ml conical centrifuge tubes. Fluorescein labeled monoclonal antibodies with specificity for the human lymphocyte surface markers CD2 (AMAC, Westbrook, Me.), CD20 (Becton Dickinson) and human IgM (Binding Site, San Diego, Calif.) were added to 3 of the tubes for identifying T and B lymphocyte populations. All reagents had previously been tested positive to the corresponding monkey lymphocyte antigens. Chimeric anti-CD20 antibody bound to monkey B cell surface CD20 was measured in the fourth tube using polyclonal goat anti-human IgG coupled with phycocerythrin (AMAC). This reagent was pre-adsorbed on a monkey Ig-sepharose column to prevent cross-reactivity to monkey Ig, thus allowing specific detection and quantitation of chimeric anti-CD20 antibody bound to cells. A fifth tube included both anti-IgM and anti-human IgG reagents for double stained B cell population. A sixth sample was included with no reagents for determination of autofluorescence. Cells were incubated with fluorescent antibodies for 30 min., washed and fixed with 0.5 ml of fixation buffer (0.15M NaCl, 1% paraformaldehyde, pH7.4) and analyzed on a Becton Dickinson FACScant™ Instrument. Lymphocyte populations were initially identified by forward versus right angle light scatter in a dot-plot bitmap with unlabeled leukocytes. The total lymphocyte population was then isolated by gating out all other events. Subsequent fluorescence measurements reflected only gated lymphocyte specific events.

Depletion of Peripheral Blood B Lymphocytes

No observable difference could be ascertained between the efficacy of CHO and SP2/0 produced antibodies in depleting B cells in vivo, although a slight increase in B cell recovery beginning after day 7 for monkeys injected with chimeric anti-CD20 antibodies derived from CHO transfectomas at dosage levels 1.6 mg/kg and 6.4 mg/kg was observed and for the monkey injected with SP2/0 producing antibody at the 0.4 mg/kg dose level. FIGS. 9A, B and C provide the results derived from the chimeric anti-CD20:CHO & SP2/0 study, with FIG. 9A directed to the 0.4 mg/kg dose level; FIG. 9B directed to the 1.6 mg/kg dose level; and FIG. 9C directed to the 6.4 mg/kg dose level.

As is evident from FIGS. 9A–C, there was a dramatic decrease (>95%) in peripheral B cell levels after the therapeutic treatment across all tested dose ranges, and these levels were maintained up to seven (7) days post-infusion; after this period, B cell recovery began, and, the time of recovery initiation was independent of dosage levels.

In the Chimeric Anti-CD20:CHO study, a 10-fold lower antibody dosage concentration (0.01 mg/kg) over a period of four daily injections (0.04 mg/kg total) was utilized. FIG. 10 provides the results of this study. This dosage depleted the peripheral blood B cell population to approximately 50% of normal levels estimated with either the anti-surface IgM or the Leu 16 antibody. The results also indicate that satiruation of the CD20 antigen on the B lymphocyte population was not achieved with immunologically active chimeric anti-CD20 antibody at this dose concentration over this period of time for non-human primates; B lymphocytes coated with the antibody were detected in the blood samples during the initial three days following therapeutic treatment. However, by day 7, antibody coated cells were undetectable.

Table I summarizes the results of single and multiple doses of immunologically active chimeric anti-CD20 antibody on the peripheral blood populations; single dose condition was 6.4 mg/kg; multiple dose condition was 0.4 mg/kg over four (4) consecutive days (these results were derived from the monkeys described above).

<table>
<thead>
<tr>
<th>TABLE I</th>
</tr>
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<tbody>
<tr>
<td>PERIPHERAL BLOOD POPULATION FROM CHO FEMALE MONKEY</td>
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</tr>
<tr>
<td>A</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
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<tr>
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<td></td>
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<tr>
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<table>
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<tr>
<th>Monkey</th>
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<th>Anti-Hu IgM*</th>
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<tbody>
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*Double staining population which indicates extent of chimeric anti-CD20 coated B cells.

The data summarized in Table I indicates that depletion of B cells in peripheral blood under conditions of antibody excess occurred rapidly and effectively, regardless of single or multiple dosage levels. Additionally, depletion was observed for at least seven (7) days following the last injection, with partial B cell recovery observed by day 21.

Table II summarizes the effect of immunologically active, chimeric anti-CD20 antibodies on cell populations of lymph nodes using the treatment regimen of Table I (4 daily doses
of 0.4 mg/kg; 1 dose of 6.4 mg/kg); comparative values for normal lymph nodes (control monkey, axillary and inguinal) and normal bone marrow (two monkeys) are also provided.

<table>
<thead>
<tr>
<th>TABLE II</th>
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<th>Anti-Hu IgM</th>
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<td>D</td>
<td>69.9</td>
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<td>74.1</td>
<td>19.9</td>
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The results of Table II evidence effective depletion of B lymphocytes for both treatment regimens. Table II further indicates that for the non-human primates, complete saturation of the B cells in the lymphatic tissue with immunologically active, chimeric anti-CD20 antibody was not achieved; additionally, antibody coated cells were observed seven (7) days after the marker depletion of lymph node B cells, observed on day 14.

Based upon this data, the single High Dosage Chimeric Anti-CD20 study referenced above was conducted, principally with an eye toward pharmacology/toxicology determination. In this study was conducted to evaluate any toxicity associated with the administration of the chimeric antibody, as well as the efficacy of B cell depletion from peripheral blood lymph nodes and bone marrow. Additionally, because the data of Table II indicates that for that study, the majority of lymph node B cells were depleted between 7 and 14 days following treatment, a weekly dosing regimen might evidence more efficacious results. Table III summarizes the results of the High Dosage Chimeric Anti-CD20 study.

<table>
<thead>
<tr>
<th>TABLE III</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Monkey</th>
<th>CD2</th>
<th>Anti-Hu IgM</th>
</tr>
</thead>
<tbody>
<tr>
<td>E</td>
<td>0.4 mg/kg</td>
<td>Day</td>
</tr>
<tr>
<td>F</td>
<td>6.4 mg/kg</td>
<td>14</td>
</tr>
<tr>
<td>G</td>
<td>0.4 mg/kg</td>
<td>28</td>
</tr>
<tr>
<td>H</td>
<td>0.4 mg/kg</td>
<td>14</td>
</tr>
<tr>
<td>I</td>
<td>6.4 mg/kg</td>
<td>28</td>
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</table>

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inter alia, the impact of C2B8 on T-cells and B-cells. Consistently for all patients, Peripheral B Lymphocytes were depleted after infusion with C2B8 and such depletion was maintained for in excess of two weeks.

One patient (receiving 100 mg² of C2B8) evidenced a Partial Response to the C2B8 treatment (reduction of greater than 50% in the sum of the products of the perpendicular diameters of all measurable indicator lesions lasting greater than four weeks, during which no new lesions may appear and no existing lesions may enlarge); at least one other patient (receiving 500 mg/m²) evidenced a Minor Response to the C2B8 treatment (reduction of less than 50% but at least 25% in the sum of the products of the two longest perpendicular diameters of all measurable indicator lesions). For presentational efficiency, results of the PBLs are set forth in Figs. 14A and B; data for the patient evidencing a PR is set forth in FIG. 14A, for the patient evidencing an M.R. data is set forth in FIG. 14B. In FIGS. 14A and B, the following are applicable: •=Lymphocytes; ⊕=CD3+ cells (T cells); □=CD20+ cells; □=CD19+ cells; □=Kappa; □=Lamabda; and =C2B8. As evidenced, the B cell markers CD20 and CD19, Kappa and Lambda, were depleted for a period in excess of two weeks; while there was a slight, initial reduction in T-cell counts, these returned to an approximate base-line level in a relatively rapid time-frame.

ii. Phase I/II Clinical Trial of C2B8: Multiple Dose Therapy Study

Patients having histologically confirmed B cell lymphoma with measurable progressive disease are eligible for this study which is separated into two parts: in Phase I, consisting of a dose escalation to characterize dose limiting toxicities and determination of biologically active tolerated dose level, groups of three patients will receive weekly i.v. infusions of C2B8 for a total of four (4) separate infusions. Cumulative dose at each of the three levels will be as follows: 500 mg/m² (125 mg/m²/infusion); 1000 mg/m² (250 mg/m²/infusion); 1500 mg/m² (375 mg/m²/infusion). A biologically active tolerated dose is defined, and will be determined, as the lowest dose with both tolerable toxicity and adequate activity; in Phase II, additional patients will receive the biologically active tolerated dose with an emphasis on determining the activity of the four doses of C2B8.

IV. COMBINATION THERAPY: C2B8 AND Y2B8

A combination therapeutic approach using C2B8 and Y2B8 was investigated in a mouse xenograft model (nu/nu mice. female. approximately 10 weeks old) utilizing a B cell lymphoblastic tumor (Ramos tumor cells). For comparative purposes, additional mice were also treated with C2B8 and Y2B8.

Ramos tumor cells (ATCC. CRL. 1596) were maintained in culture using RPMI-1640 supplemented with 10% fetal calf serum and glutamine at 37°C. C. 5% CO₂. Tumors were initiated in nine female nude mice approximately 7-10 weeks old by s.c. injection of 1.7×10⁸ Ramos cells in a volume of 0.10 ml (HBSS) using a 1 cc syringe fitted with 25 g needle. All animals were maintained in a laminar flow hood and all cages, bedding, food and water were autoclaved. Tumor cells were passaged by excising tumors and passing these through a 40 mesh screen; cells were washed twice with HBSS (50 ml) by centrifugation (1300 RPM), resuspended in 1× HBSS to 10×10⁶ cells/ml, and frozen at -70°C until used.

For the experimental conditions, cells from several frozen lots were thawed, pelleted by centrifugation (1300 RPM) and washed twice with HBSS. Cells were then resuspended to approximately 2.0×10⁶ cells/ml. Approximately 9 to 12 mice were injected with 0.10 ml of the cell suspension (s.c.) using a 1 cc syringe fitted with a 25 g needle; injections were made on the animal’s left side, approximately mid-region. Tumors developed in approximately two weeks. Tumors were excised and processed as described above. Study mice were injected as described above with 1.67×10⁶ cells in 0.10 ml HBSS.

Based on preliminary dosing experiments, it was determined that 200 mg of C2B8 and 100 μCi of Y2B8 would be utilized for the study. Ninety female nu/nu mice (approximately 10 weeks old) were injected with the tumor cells. Approximately ten days later, 24 mice were assigned to four study groups (six mice/group) while attempting to maintain a comparable tumor size distribution in each group (average tumor size, expressed as a product of length x width of the tumor, was approximately 80 mm³). The following groups were treated as indicated via tail-vein injections using a 100 μl Hamilton syringe fitted with a 25 g needle:

A. Normal Saline
B. Y2B8 (100 μCi)
C. C2B8 (200 μg); and
D. Y2B8 (100 μCi)+C2B8 (200 μg)

Groups treated with C2B8 were given a second C2B8 injection (200 μg/mouse) seven days after the initial injection. Tumor measurements were made every two or three days using a caliper.

Preparation of treatment materials were in accordance with the following protocols:

A. Preparation of Y2B8

Yttrium-90(90Y) chloride was transformed into a polycarbonate tube and adjusted to pH 4.1-4.4 using metal free 2M sodium acetate, 2B8-MX-DTPA (0.3 mg in normal saline; see above for preparation of 2B8-MX-DTPA) was added and gently mixed by vortexing. After 15 min incubation, the reaction was quenched by adding 0.05× volume 20 mM EDTA and 0.05× volume 2M sodium acetate. Radioactivity concentration was determined by diluting 5.0 μl of the reaction mixture in 2.5 ml PBS containing 75 mg/ml HSA and 1 M DTPA (“Formulation buffer”); counting was accomplished by adding 10.0 μl to 20 ml of Eclatec™ scintillation cocktail. The remainder of the reactive mixture was added to 3.0 ml formulation buffer, sterile filtered and stored at 2°C-8°C until used. Specific activity (14 μCi/mg at time of injection) was calculated using the radioactivity concentration and the calculated protein concentration based upon the amount of antibody added to the reaction mixture. Protein-associated radioactivity was determined using instant thin-layer chromatography, Radiolincorporation was 95%. Y2B8 was diluted in formulation buffer immediately before use and sterile filtered (final radioactivity concentration was 1.0 μCi/ml).

B. Preparation of C2B8

C2B8 was prepared as described above. C2B8 was provided as a sterile reagent in normal saline at 5.0 mg/ml. Prior to injection, the C2B8 was diluted in normal saline to 2.0 mg/ml and sterile filtered.

C. Results

Following treatment, tumor size was expressed as a product of length and width, and measurements were taken on the days indicated in FIG. 11 (Y2B8 vs. Saline); FIG. 12 (C2B8 vs. Saline); and FIG. 13 (Y2B8+C2B8 vs. Saline). Standard error was also determined.

As indicated in FIG. 13, the combination of Y2B8 and C2B8 exhibited tumoricidal effects comparable to the effects evidenced by either Y2B8 or C2B8.

V. ALTERNATIVE THERAPY STRATEGIES

Alternative therapeutic strategies recognized in view of the foregoing examples are evident. One such strategy
employs the use of a therapeutic dose of C2B8 followed within about one week with a combination of either 2B8 and radiolabeled 2B8 (eg, Y2B8); or 2B8, C2B8 and, eg, Y2B8. An additional strategy is utilization of radiolabeled C2B8—such a strategy allows for utilization of the benefits of the immunologically active portion of C2B8 plus those benefits associated with a radiolabel. Preferred radiolabels include yttrium-90 given the larger circulating half-life of C2B8 versus the murine antibody 2B8. Because of the ability of C2B8 to deplete B-cells, and the benefits to be derived from the use of a radiolabel, a preferred alternative strategy is to treat the patient with C2B8 (either with a single dose or multiple doses) such that most, if not all peripheral B cells have been depleted. This would then be followed with the use of radiolabeled 2B8; because of the depletion of peripheral B cells, the radiola belled 2B8 stands an increased chance of targeting tumor cells. Iodine [131] labeled 2B8 is preferably utilized, given the types of results reported in the literature with this label (see Kaminski). An alternative strategy involves the use of a radiolabeled 2B8 (or C2B8) first in an effort to increase the permeability of a tumor, followed by single or multiple treatments with C2B8; the intent of this strategy is to increase the chances of the C2B8 in getting both outside and inside the tumor mass. A further strategy involved the use of chemotherapeutic agent in combination with C2B8. These strategies include so-called “staggered” treatments, ie, treatment with chemotherapeutic agent, followed by treatment with C2B8, followed by a repetition of this protocol. Alternatively, initial treatment with a single or multiple doses of C2B8, thereafter followed with chemotherapeutic treatment, is viable. Preferred chemotherapeutic agents include, but are not limited to: cyclophosphamide; doxorubicin; vincristine; and prednisone. See Armitage, J. O. et al., Cancer 50:1695 (1982), incorporated herein by reference.

The foregoing alternative therapy strategies are not intended to be limiting, but rather are presented as being representative.

VL DEPOSIT INFORMATION

Anti-CD20 in TCAE 8 (transformed in E. coli for purposes of deposit) was deposited with the American Type Culture Collection (ATCC) on Nov. 4, 1992, 12301 Parklawn Drive, Rockville, Md., 20852, under the provisions of the Budapest Treaty for the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure ("Budapest Treaty"). The microorganism was tested by the ATCC on Nov. 9, 1992, and determined to be viable on that date. The ATCC has assigned this microorganism for the following ATCC deposit number: ATCC 69119 (anti-CD20 in TCAE 8). Hybridoma 2B8 was deposited with the ATCC on Jun. 22, 1993 under the provisions of the Budapest Treaty. The viability of the culture was determined on Jun. 25, 1993 and the ATCC has assigned this hybridoma the following ATCC deposit number: HB 11388.

5,736,137
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(2) INFORMATION FOR SEQ ID NO:3:

(1) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9399 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: circular

(1) MOLECULE TYPE: DNA (generic)

(3) HYPOTHETICAL: NO

(4) ANTI-SENSE: NO

(5) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 47 base pairs
(B) TYPE: nucleic acid
(C) STRANDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:
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(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:
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G T C ATA ATG TCC AAG GGG CAA ATT GTT TCC CAA CAG TCT CCA GCA ATC
Val Ile Met Ser Arg Gly Glu Ile Val Leu Ser Gin Ser Pro Ala Ile
-5 .1 1 5 10

C T G C T TCA TTT CCA GGG CAG AAC GTC ACA ATG ACT TGC AGG GCC AGC
Leu Ser Ala Ser Pro Gin Glu Lys Val Thr Met Thr Cys Arg Ala Ser
-15 20 25

T C A ACT GTC AGT TAC ATC CAC TGG TCC CAG CAG AAG CCA GGA TCC
Ser Ser Val Ser Tyr Ile His Trp Phe Gin Gin Lys Pro Gin Ser Ser
35 40

C C C AAA CCC TGG ATT TAT GCC ACA TCC AAC CTG GCT TCT GGA GTT CCT
Pro Lys Pro Trp Ile Tyr Ala Thr Ser Asn Leu Ala Ser Gin Val Pro
45 50 55

G T T CGC TTC AGT GGC AGT GGG TCT GGG ACT TCT TAC TCT CTC ACA ATC
Val Arg Phe Ser Gin Ser Gin Ser Gin Ser Gin Ser Gin Ser Gin Ser
60 65 70

A G C AGA GTC GAG GCT GAA GAT GCT GCC ACT TAT TAC TGC CAG CAA TGG
Ser Arg Val Glu Ala Gin Asp Ala Ala Thr Tyr Tyr Cys Gin Gin Trp
75 80 85 90

A C T AGT AAC CCA CCC ACG TTC GGA GGG GGG ACC AAG CTC GAA ATC AAA
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if py 5,736,137

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INFORMATION FOR SEQ ID NO:8:

SEQUENCE CHARACTERISTICS:
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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

MOLECULE TYPE: DNA (genomic)

HYPOTHETICAL: NO

ANTI-SENSE: YES

FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 3
(D) OTHER INFORMATION: /note="Nucleotide 5 is N wherein N is G or C."

FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 18
(D) OTHER INFORMATION: /note="Nucleotide 18 is N wherein N is A or C."

FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 25
(D) OTHER INFORMATION: /note="Nucleotide 25 is N wherein N is G or A."

SEQUENCE DESCRIPTION: SEQ ID NO:

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INFORMATION FOR SEQ ID NO:9:

SEQUENCE CHARACTERISTICS:
(A) LENGTH: 420 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

MOLECULE TYPE: DNA (genomic)

HYPOTHETICAL: NO

ANTI-SENSE: NO

FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1-420

FEATURE:
(A) NAME/KEY: mac_peptide
(B) LOCATION: 51-420

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Val Leu Ser Gln Val Gln Leu Gln Gln Pro Gly Ala Glu Leu Val Lys
-1 -10 -5

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What is claimed is:

1. Immunologically active, chimeric anti-CD20 antibody produced from a transfectoma comprising anti-CD20 in TCAE 8, ATCC deposit number 69119.

2. A pharmaceutical composition comprising the anti-CD20 antibody of claim 1 in a pharmaceutically acceptable carrier.

3. The composition of claim 2, which contains a pharmaceutically acceptable carrier or excipient selected from the group consisting of sterile saline, sterile buffered water, propylene glycol and mixtures thereof.

4. The composition of claim 2, which contains a pharmaceutically acceptable dosage of the antibody which ranges from about 0.001 to about 30 mg/kg of human body weight.

5. The composition of claim 4, which contains a pharmaceutically acceptable dosage of the antibody which ranges from about 0.01 to about 25 mg/kg human body weight.

6. The composition of claim 5, which contains a pharmaceutically acceptable dosage of the antibody which ranges from about 0.4 to about 26.0 mg/kg human body weight.

* * * * *
UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO. : 5,736,137
APPLICATION NO. : 08/149099
DATED : April 7, 1998
INVENTOR(S) : D. R. Anderson et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

On the Title page of the patent, the identification of Inventors [75] should read:

-- Darrell R. Anderson, Escondido;
   Nabil Hanna, Olivenhain;
   Roland A. Newman;
   Mitchell E. Reff, both of San Diego;
   William H. Rastetter, Rancho Sante Fe, all of Calif. --

Signed and Sealed this

Twenty-third Day of June, 2009

[Signature]

JOHN DOLL
Acting Director of the United States Patent and Trademark Office
UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO. : 5,736,137
APPLICATION NO. : 08/149099
DATED : April 7, 1998
INVENTOR(S) : D. R. Anderson et al.

Signed and Sealed this Twenty-ninth Day of March, 2016
Michelle K. Lee
Director of the United States Patent and Trademark Office

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Delete Drawing Sheet 15 of 21 and substitute therefore with the attached Drawing Sheet 15 of 21 consisting of FIG. 5
FIG. 5